The Inhibition of Mucopeptide Synthesis by Benzylpenicillin in Relation to Irreversible Fixation of the Antibiotic by Staphylococci

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1. Benzylpenicillin is irreversibly fixed to staphylococci by a reaction that obeys second-order kinetics, whereas the progress of inhibition of mucopeptide synthesis obeys first-order kinetics after a short lag during which the antibiotic has no effect.
2. When the micro-organisms are saturated with benzylpenicillin they can still make mucopeptide in solutions containing chloramphenicol at a normal rate after a lag period.
3. About 90% of the benzylpenicillin stays fixed to the cells after mucopeptide synthesis has reached its maximum and constant rate.
4. During the phase when mucopeptide synthesis by cells saturated with benzylpenicillin is accelerating, a small number of additional sites that fix benzylpenicillin is revealed. The number of these sites reaches a maximum and constant value at about the same time as mucopeptide biosynthesis reaches a maximum and constant rate.
5. Staphylococci saturated with benzylpenicillin are exceedingly sensitive to fresh additions of the antibiotic.
6. The degree of inhibition of mucopeptide synthesis caused by these small amounts of antibiotic agrees with the degree of substitution by benzylpenicillin of the newly revealed or 'sensitive' sites.
7. Since these sensitive sites are revealed during incubation of the bacteria with chloramphenicol it is unlikely that they are due to newly formed protein.
8. On the basis of these results, a hypothesis for the inhibition by penicillin of the cross-linking reaction in the terminal stages of mucopeptide synthesis is suggested.

It is now accepted that an early and important step in the action of penicillins is to inhibit the proper formation of the structurally unique class of polymers, the mucopeptides, in the walls of bacteria. As an immediate consequence of this inhibition the formation of other polymers in the wall such as the ribitol teichoic acid in staphylococci is also inhibited (Rogers & Garrett, 1965). The only step in the formation of mucopeptide by cell-free systems from staphylococci that has been shown to be affected by the penicillins is a late one involving the formation of cross-links between the terminal amino group of a pentaglycine chain and the carboxyl group of the subterminal alanine on a neighbouring peptide chain, as is illustrated in Fig. 1 (Wise & Park, 1965; Tipper & Strominger, 1965; Izaki, Matsuhashi & Strominger, 1966). A carboxypeptidase present in extracts of *Escherichia coli* strain B that splits the bond between the terminal and subterminal D-alanine molecules in the uridine diphosphate mucopeptide precursor has also been shown to be very sensitive to penicillin (Izaki *et al.* 1966).

Despite a number of hypotheses (Collins &

Richmond, 1962; Wise & Park, 1965; Tipper & Strominger, 1965), little is yet known about how the inhibition of cross-linking takes place. An obvious

Fig. 1. Proposed structure for incomplete mucopeptide that accumulates under some circumstances when staphylococci are treated with penicillin. The cross-linking bond is thought to be formed between the terminal glycine amino group *B* and the subterminal D-alanyl carboxyl group revealed by splitting the D-alanine-D-alanyl peptide bond at *A* in a neighbouring peptide group. MurNAc, *N*-Acetylmuramic acid; GlcNAc, *N*-acetylglucosamine.

point of departure for an attack on the problem is to examine the nature of the substances to which penicillin is irreversibly fixed in bacterial cells. Early work (Pasyanskii & Kastorskaya, 1947; Maas & Johnson, 1949; Rowley, Cooper, Roberts & Lester-Smith, 1950; Pollock & Perret, 1951; Eagle, 1954*a,b*; Daniel & Johnson, 1954; Schepartz & Johnson, 1956; Cooper, 1956) suggested that micro-organisms irreversibly fixed an amount of penicillin that was related to the sensitivity of the organisms to the antibiotic but that there were exceptions to this rule. For example, when penicillin-sensitive micro-organisms were made resistant to penicillin by selection in the laboratory, the mutants gave variable results for fixation, and sometimes no correlation between the amount of penicillin fixed and the sensitivity to the antibiotic could be found (Eagle, 1954*b*). The binding of penicillin took place in the absence of growth, occurred with dead and with disrupted organisms, appeared to be specific and was irreversible. The fixed molecules did not exchange with other penicillin molecules outside the cell and could not be removed by washing with neutral or acidic buffers or by the action of penicillinase. Only by treatment of the organisms with dilute alkali or detergents could the radioactivity of the labelled penicillin be removed.

The correlation that was usually found between the amounts fixed and the sensitivity of the bacteria to penicillin prompted the hypothesis that the sites to which the molecules were attached were important for antibiotic action. One difficulty about this idea was that the cells with their load of fixed penicillin still grew when placed in fresh growth medium (Eagle, 1954*b*). Indeed, it has long been known that the antibiotic does not affect bacteria unless they are growing. This difficulty in acceptance of the hypothesis that fixed penicillin was antibiotically important was overcome (Cooper, 1956) by supposing that the bacteria rapidly formed more of the fixation sites when placed in new medium. These new sites allowed the bacteria to grow despite the combination of the old ones with penicillin.

In disrupted staphylococci a high proportion of the penicillin appeared to be attached to particles that were made of lipid and protein (Cooper, 1954, 1955*a*). Such particles were likely to be derived from membrane structures. A more detailed examination of the site of fixation in another organism, *Bacillus cereus* (Duerksen, 1964), gave a more complicated answer. Duerksen (1964) found that 58–95% of the fixed penicillin was liberated into solution when the bacilli were converted into protoplasts by the use of lytic enzymes, leaving 7–34% attached to crude membrane material or 5% to purified membrane preparations. Most of the penicillin liberated into solution was fixed to high-

molecular-weight material (i.e. material that was non-diffusible through cellophan and excluded by Sephadex G-25). When the bacteria were mechanically disrupted after fixation of penicillin, 10–17% could be found in the crude wall fraction, but only 0.2% in a purified wall preparation, whereas 30–40% occurred in the ribosome-membrane fraction, and 40–50% could not be sedimented at 40000*g* in 2 hr. Thus it appeared that penicillin could be fixed to material in various subcellular fractions and that a high proportion was fixed to some material readily lost when the wall was removed to leave protoplasts.

In the present work the relationship between fixed benzylpenicillin and mucopeptide synthesis is studied under conditions where no more protein can be formed and, of course, growth cannot occur. The earlier hypothesis (see Cooper, 1956) that all the penicillin fixed to bacteria was important as antibiotic was never strong, and the evidence now presented suggests that most of it is irrelevant as far as mucopeptide synthesis is concerned. A preliminary account of this work has been published elsewhere (Rogers, 1966*a*). A small number of penicillin-binding sites have been found, however, that do seem to be important for mucopeptide biosynthesis, since combination of these with benzylpenicillin inhibits the process. In the light of these results a new hypothesis for the action of penicillin is suggested.

Before undertaking studies of the effects of fixed benzylpenicillin on staphylococci it was necessary to examine the validity of methods to be used for the estimation of mucopeptide biosynthesis. There are a number of discrepancies in the published studies of mucopeptide biosynthesis by whole bacteria, particularly about the sensitivity of the process to benzylpenicillin.

Most of these differences appear to be due to the different techniques used for measuring mucopeptide synthesis or to the use of different strains of bacteria. For example, the difference between the result obtained by Mandelstam & Rogers (1959) and the later results from this Laboratory can be explained by the use in the earlier work of a penicillinase-forming strain (Rogers, 1962) and by measurement of the increase in whole wall material that included teichoic acid (Rogers & Garrett, 1965). On the other hand, the initial lag in the action of benzylpenicillin observed by Rogers & Jeljaszewicz (1961) appears, from the results given in the present paper, to be due to incorporation of radioactive amino acids into material that behaves differently from mucopeptide. Confusion by this initial lag can be avoided by measuring the amount of amino acid incorporated into the mucopeptide rather than into all the material insoluble in hot trichloroacetic acid. Meanwhile, comparison of the chemically measured

amounts of mucopeptide synthesized with the incorporation of radioactively labelled amino acids has shown that in the presence of penicillin significant turnover of the mucopeptide in the walls of staphylococci occurs.

MATERIALS

[9-¹⁴C]Benzylpenicillin. This was as supplied by The Radiochemical Centre, Amersham, Bucks., and was labelled with ¹⁴C in the carbonyl group in the side chain (i.e. C-9 of the benzylpenicillin molecule). Three batches of material were obtained, each having a specific activity of 15.7 mc/m-mole. The material was dissolved in 0.01 M-phosphate buffer, pH 7.0, to give 1.0 mg./ml., and was kept frozen at -20°.

Unlabelled benzylpenicillin. This was Crystapen, as supplied by Glaxo Laboratories, Greenford, Middx. Solutions of 1 mg./ml. or 10 mg./ml. were made freshly in 0.01 M-phosphate buffer, pH 7.0, for each experiment and diluted appropriately in the same buffer.

Other chemicals. [1-¹⁴C]Glycine and [1-¹⁴C]glutamic acid were as supplied by The Radiochemical Centre, and were dissolved in water to give solutions containing 10 µc/ml.

Micro-organisms. *Staphylococcus aureus* strain Oxford was used for all the work described. The minimum growth-inhibitory concentration of benzylpenicillin for the non-penicillinase-forming strain is 0.04–0.05 µg./ml.

METHODS

Growth of micro-organisms and preparation of suspensions. The bacteria were grown as by Rogers & Garrett (1965). Suspensions were prepared to contain 1 or 2 mg./ml. dry wt. of cells in either the phosphate–glucose buffer described by Mandelstam & Rogers (1959) or in 0.1 M-phosphate buffer, pH 7.0, made by mixing the appropriate volumes of equimolar solutions of KH₂PO₄ and Na₂HPO₄.

Measurement of mucopeptide synthesis. Two methods were used: (i) determination of the incorporation of [1-¹⁴C]glycine into samples of mucopeptide prepared according to Park & Hancock (1960); (ii) measurement of the increase in the amount of mucopeptide prepared by the modified Park & Hancock (1960) method by chemical means. The amount of mucopeptide was measured by hydrolysing a suitable sample with 6 N-HCl overnight (about 16 hr.) at 105°, removing the acid *in vacuo* by repeated addition of water to the dried material, and determining amino nitrogen by an adaptation of the ninhydrin method (Jacobs, 1956). The conditions for incorporation of radioactivity into the mucopeptide of the cell walls were as previously described (Mandelstam & Rogers, 1959; Rogers & Garrett, 1965). Chloramphenicol was always present at a concentration of 50 µg./ml.

Preparation of mucopeptide. This was done by a modification of the Park & Hancock (1960) method in which the NH₄HCO₃–aq. NH₃ buffer was replaced by 0.1 M-sodium–potassium phosphate at pH 7.6 during trypsin digestion and the amount of enzyme used was raised to 1 mg./ml. The precipitate, insoluble in hot trichloroacetic acid, was washed with the phosphate buffer before the enzymic digestion.

Fixation of [9-¹⁴C]benzylpenicillin. The cell suspension

in 0.1 M-sodium–potassium phosphate buffer or in glucose phosphate buffer (Mandelstam & Rogers, 1959) was left at a suitable temperature with the [9-¹⁴C]benzylpenicillin. Experiments showed no difference between the amounts fixed with the two buffers, provided that the temperature was 37° or less and the time not longer than 30 min. At appropriate times either the cells were immediately removed by centrifuging and washed twice with an equal volume of buffer, or a sample of the suspension was added to 0.1 vol. of unlabelled benzyl-penicillin solution (10 mg./ml.) so as to dilute the radioactive material usually present at 0.3–1.0 µg./ml. to the point where further fixation of radioactivity was not perceptible. The cells were then washed once with a solution of unlabelled benzylpenicillin (1 mg./ml.) dissolved in 0.1 M-sodium–potassium phosphate buffer, pH 7.0, and once with water. The deposits of bacteria were finally suspended in 0.5 ml. volumes of water and 0.2 ml. samples counted in a liquid scintillation counter. The number of counts obtained by suspending the whole bacteria in scintillating fluid was the same as was obtained by dissolving the bacteria by mild acid hydrolysis and counting the hydrolysate after neutralization. In some experiments samples were counted as solids in a solid-sample counter, by drying 5–10 mg. dry wt. from the frozen state on a 0.3 cm.² planchet.

Radioactive counting techniques. Samples were counted by using: (i) The Packard Tri-Carb scintillation counter. The scintillation fluid used was a solution of 18% (w/v) naphthalene, 0.4% 2,5-diphenyloxazole, 0.01% 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene dissolved in dioxan; 10 ml. of scintillation fluid was added in the standard vial. Correction for background and quenching were applied and more than 1000 counts above background were always collected. (ii) The Nuclear–Chicago windowless gas-flow counter. Corrections were made for background and self-absorption. More than 1000 counts above background were collected. All results are expressed as counts/min., disintegrations/min., or weight of material, according to which was appropriate.

RESULTS

Techniques for measuring the inhibition of mucopeptide synthesis by benzylpenicillin

Incorporation into material insoluble in hot trichloroacetic acid. This technique was the one used by Rogers & Jeljaszewicz (1961). In the present experiment cells incorporated [1-¹⁴C]glutamic acid in the usual system with chloramphenicol present, in the presence and absence of 3 µg. of benzylpenicillin/ml. for 1 hr. At the end of this time the material insoluble in 5% (w/v) trichloroacetic acid at 90° was isolated, washed with 5% (w/v) trichloroacetic acid containing 1 mg. of unlabelled glutamic acid/ml. and then, successively, with 5% (w/v) trichloroacetic acid, water, ethanol and ether, and the radioactivity of the dry material measured. As judged from the radioactivity in this material, 3 µg. of benzylpenicillin/ml. caused 64% inhibition of incorporation. The insoluble material was then suspended in 1.0 ml. of 0.05 N-hydrochloric acid and 2 mg. of crystalline pepsin added. A few drops of toluene were added and the suspensions were

incubated at 35° overnight. The insoluble and soluble parts were then separated, the former being washed twice with water and the radioactivity in both counted. Of the counts in the original material insoluble in hot trichloroacetic acid, 67% were removed when benzylpenicillin was present and only 17% from the control cells. All the available evidence shows that fully formed mucopeptide is not attacked by pepsin (e.g. Salton, 1964; H. J. Rogers & H. R. Perkins, unpublished work). The radioactive material removed by pepsin is therefore not likely to be part of fully formed mucopeptide. When allowance is made for the radioactive material removed by pepsin, the degree of inhibition by the benzylpenicillin of the synthesis of fully formed mucopeptide becomes 86%. This result agrees fairly well with that of 90–95% inhibition obtained by Rogers & Garrett (1965) after incorporation of [^{14}C]alanine into mucopeptide. Thus it appears that the incomplete inhibition and probably the lag in action of penicillin observed by Rogers & Jeljaszewicz (1961) is due to incorporation of the amino acids into some material other than fully formed mucopeptide.

Incorporation into isolated mucopeptide. A large number of experiments have now been done by using the Park & Hancock (1960) method for isolating mucopeptide, and the two experiments quoted in Table 1 are those that showed the greatest variation in degree of inhibition by benzylpenicillin. However, even when relatively low degrees of inhibition were found by this method (e.g. in the second experiment, Table 1), these could be raised to

90% or more by increasing the benzylpenicillin concentration. There was no evidence for an initial lag in the action of benzylpenicillin except at very low concentrations of the antibiotic (i.e. less than 0.05 $\mu\text{g./ml.}$).

Turnover of mucopeptide in staphylococci inhibited by benzylpenicillin. When mucopeptide synthesis by suspensions of staphylococci incubated with benzylpenicillin was measured chemically and by incorporation of any of the four amino acids present in mucopeptide, it was found that the results did not completely agree. There was a slow incorporation of radioactivity into mucopeptide that was not matched by an increase in the total amount of mucopeptide in the cells. In the experiment shown in Fig. 2, in which the cells were incubated with 2 $\mu\text{g.}$ of benzylpenicillin/ml., there was a slow loss of mucopeptide, despite a continued incorporation of radioactivity. As is shown elsewhere (Rogers, 1966b) and in Fig. 3(a), a concentration of the penicillins can be chosen that just keeps the amount of mucopeptide associated with the cells constant. With benzylpenicillin this concentration has been found to be about 0.3 $\mu\text{g./ml.}$ (see Figs. 3a and 3b). As the concentration of antibiotic is increased an inhibition of the synthesis side of the balanced turnover takes place (Rogers, 1966b). Thus a total inhibition of net synthesis does not mean that all synthesis is completely inhibited. This incorporation by turnover is small relative to that in the control culture (Fig. 2), and is unlikely to confuse the picture except in situations where synthesis is strongly inhibited. Because it is such a small proportion of the total in an uninhibited system, attempts to show turnover of mucopeptide in the absence of benzylpenicillin are likely to be difficult. It cannot therefore be said at present with certainty whether the penicillins provoke the turnover or simply reveal it. However, more detailed experiments (H. J. Rogers, unpublished work) have shown conclusively that a turnover of the mucopeptide can occur in the presence of penicillin.

As a result of this work it was decided that the incorporation of radioactively labelled amino acids into the material isolated by the Park & Hancock (1960) method provided a valid estimation of the biosynthesis of the mucopeptide. This method, applied in a large number of experiments over a period of 4 years, has shown that 0.05 $\mu\text{g.}$ of benzylpenicillin added at the beginning of the incubation of a suspension (1 mg./ml.) of the Oxford strain of *Staph. aureus* in the suspension fluid always causes 35–60% inhibition of synthesis after 1 hr. incubation. A concentration of benzylpenicillin of 1.0–3.0 $\mu\text{g./ml.}$ causes 90–95% inhibition. In any given experiment with the same suspension of microorganisms the degree of inhibition between duplicates agrees to well within 10%. No instance of the

Table 1. *Effect of benzylpenicillin on the biosynthesis of mucopeptide by Staph. aureus strain Oxford*

The cells were grown, suspensions prepared and then incubated as described in the Methods section. At the end of 1 hr. samples were taken into trichloroacetic acid (final concn. 5%, w/v) and mucopeptide was prepared by the modified Park & Hancock (1960) method. Radioactivity determinations were made with the Nuclear-Chicago windowless gas-flow counter and are given as counts/min. N.E., Not examined. Expts. 1 and 2 were made with an interval of 2 years between them.

Concn. of benzyl- penicillin ($\mu\text{g./ml.}$)	Total radioactivity in mucopeptide from 1 mg. dry wt. of bacteria (counts/min.)		Inhibition (%)	
	Expt. 1	Expt. 2	Expt. 1	Expt. 2
0	309	350	—	—
0.02	253	N.E.	18	N.E.
0.05	127	206	59	38
0.10	80.5	170	69	51
0.50	22.2	N.E.	93	N.E.
1.0	22.9	91	93	74

extreme insensitivity observed by Wise & Park (1965) has been found. For some reason the incomplete mucopeptide that is formed in the

presence of benzylpenicillin stays associated with the insoluble mucopeptide in the experiments of Wise & Park (1965) but fails to do so in my system. Net synthesis of mucopeptide, as measured chemically in a smaller number of experiments, is 95–100% inhibited by 0.3–0.4 $\mu\text{g.}$ of benzylpenicillin.

Fig. 2. Comparison of incorporation of $[1-^{14}\text{C}]$ glycine with the chemically measured amount of mucopeptide. The Figure shows the amount of radioactivity incorporated in the presence ($\circ$ — $\circ$) and absence ($\triangle$ — $\triangle$) of 2 $\mu\text{g.}$ of benzylpenicillin/ml., and the amounts of mucopeptide estimated chemically in the presence ($\bullet$ — $\bullet$) and absence ($\blacktriangle$ — $\blacktriangle$) of antibiotic. The conditions of incubation were the usual ones in which a suspension (1 mg./ml.) of *Staph. aureus* strain Oxford was incubated at 35° with the four amino acids present in the mucopeptide, $[1-^{14}\text{C}]$ glycine and chloramphenicol. Samples (3.0 ml.) were taken at the indicated times and mucopeptide was prepared.

Irreversible fixation of benzylpenicillin

Kinetics of fixation. The kinetics of the fixation of $[9-^{14}\text{C}]$ benzylpenicillin at three temperatures is shown in Fig. 4. The process obeys strictly the kinetics of a second-order reaction. Both the concentration of benzylpenicillin molecules and the number of reactive sites in the cell may be important in defining the speed of the reaction. As is shown by the results in Fig. 4, the reaction is temperature-sensitive. In view of the subsequent results it should be pointed out that one of the characteristics of a second-order reaction is that the half-life of the reactants does not remain constant but increases as the reaction proceeds, i.e. as the cell becomes saturated with benzylpenicillin molecules the speed at which the remaining sites become covered will decrease.

Saturation of the irreversible sites. When suspensions of staphylococci containing 1 mg. dry wt. of bacteria/ml. were incubated at 20° with varying concentrations of $[9-^{14}\text{C}]$ benzylpenicillin for 30 min. and then treated with an excess of unlabelled antibiotic, washed and the amount of radioactivity they contained was estimated, curves of the type shown in Fig. 5 were obtained. The batches of $[9-^{14}\text{C}]$ benzylpenicillin used were different deliveries having the same batch number and specific activity.

Fig. 3. Effects on mucopeptide biosynthesis occurring in growing cultures of benzylpenicillin added together with chloramphenicol. (a) Effect on the increase in the amount of mucopeptide estimated chemically. (b) Effect on the incorporation of $[1-^{14}\text{C}]$ glycine when trace amounts were added at the same time as the benzylpenicillin and chloramphenicol. $\bullet$, Benzylpenicillin absent; $\circ$, benzylpenicillin (0.3 $\mu\text{g./ml.}$) present. In this experiment the bacteria were allowed to grow to a density of 0.06 mg./ml. in a casein-hydrolysate medium, and the chloramphenicol (50 $\mu\text{g./ml.}$ of culture) was added to both and benzylpenicillin (0.3 $\mu\text{g./ml.}$ of culture) added to one of these. Samples (10 ml.) were taken into 1.0 ml. of 50% (w/v) trichloroacetic acid, and mucopeptide was prepared.

Fig. 4. Second-order kinetics of irreversible fixation of penicillin. In the logarithmic plot of $(a-x)/(b-x)$ against time, a represents the initial concentration ($0.2 \mu\text{g./ml.}$) of $[9\text{-}^{14}\text{C}]$ benzylpenicillin (expressed as counts/min./ml.), b the concentration of fixation sites/ml. of suspension (expressed as counts/mg. dry wt. of bacteria fully saturated with $[9\text{-}^{14}\text{C}]$ benzylpenicillin), and x the counts/mg. dry wt. of bacteria in the samples taken during the experiment. The suspensions contained 1 mg. dry wt. of bacteria/ml. The experiments were done at three temperatures: $\bullet$, 37° ; $\circ$, 20° ; $\triangle$, 0° .

Fig. 5. Saturation of the bacteria with $[9\text{-}^{14}\text{C}]$ benzylpenicillin. Suspensions (1 mg./ml.) of *Staph. aureus* strain Oxford in 0.1M-phosphate buffer, pH 7.0, were treated with different concentrations of $[9\text{-}^{14}\text{C}]$ benzylpenicillin for 30 min. at 20° . Samples were then mixed with an excess of unlabelled benzylpenicillin and treated as described in the Methods section. $\bullet$, Sample of batch 4 of $[9\text{-}^{14}\text{C}]$ benzylpenicillin delivered in December 1964; $\circ$, sample of the same batch delivered in July 1965.

The experiments were made at an interval of 8 months, during which the $[9\text{-}^{14}\text{C}]$ benzylpenicillin had been stored as a freeze-dried solid at -40° . It seems probable that the less definite saturation curve obtained at the later date is due to the presence of ^{14}C -labelled breakdown products of

benzylpenicillin in the antibiotic solution that are adsorbed to the cells along with the benzylpenicillin (cf. Pollock & Perret, 1951). The curve shown with solid symbols was with the earlier batch of presumably undegraded benzylpenicillin. From Fig. 5 it was deduced that the 'specific' irreversible fixation sites of the bacteria are more than 90% saturated when the suspension has been treated with a concentration of $0.3\text{--}0.4 \mu\text{g.}$ of benzylpenicillin/ml. The use of a minimum concentration for saturation reduces the error due to the uptake of ^{14}C not contained in benzylpenicillin.

Variation in the amount of benzylpenicillin fixed/mg. dry wt. of cells was also encountered. From nine different experiments the average amount fixed corresponded to 174 molecules/cell or $5.7 \mu\text{moles/mg.}$ dry wt. of cells and varied from 106 to 197 molecules/cell. The average number of molecules fixed/cell is lower than that obtained by earlier workers but, as was suggested by Cooper (1955b), who found a value of 300, this is no doubt due to both true variations and uncertainties in precisely estimating the number of cocci in 1 mg. dry wt. of cells. In some earlier work living counts were used to estimate the number of cocci. In the present work the number of cells in a suitable dilution of a suspension (1 mg./ml.) was counted in a haemocytometer cell (see Rogers & Spensley, 1955).

Kinetics of the inhibition of mucopeptide synthesis

To examine the kinetics of inhibition of mucopeptide biosynthesis, suspensions of the staphylococci prepared in phosphate-glucose buffer were first incubated in stationary flasks with and without $0.02 \mu\text{g.}$ of benzylpenicillin/ml. and in the absence of any amino acids. At suitable times samples were added to a concentrated solution of amino acids, $[1\text{-}^{14}\text{C}]$ glycine and chloramphenicol, to give the usual final concentrations of these substances used during the studies of mucopeptide synthesis. Incubation with shaking to aerate the suspensions was then continued for 10 min. at 20° . At the end of this time samples were taken into trichloroacetic acid, mucopeptide was prepared by the Park & Hancock (1960) procedure and the amount of radioactivity incorporated was measured. It was necessary to measure as accurately as possible the time during which benzylpenicillin reacted with the cells. Other experiments have shown that reaction goes on whether or not mucopeptide synthesis is occurring. Therefore it was desirable to keep the period of incubation with the amino acids during which mucopeptide synthesis was studied as short as possible relative to the period of incubation with benzylpenicillin in buffered glucose. This, however, raised another problem since it was found that,

Fig. 6. Kinetics of the reaction of benzylpenicillin with sites concerned with mucopeptide synthesis. A suspension (1.2 mg./ml.) of *Staph. aureus* in buffered glucose was incubated with (●) or without (○) benzylpenicillin (0.02 µg./ml.) at 20°, without agitation, for the times indicated. At the end of these times samples (8.6 ml.) were removed into 1.4 ml. of concentrated medium containing the usual four amino acids ([1-¹⁴C]glycine was included) and chloramphenicol. After 10 min. incubation at 20° with agitation, samples (5.0 ml.) were removed into 0.5 ml. of 50% (w/v) trichloroacetic acid. Mucopeptide was prepared from the cells and the radioactivity incorporated from the [1-¹⁴C]glycine estimated.

when cells were incubated in buffer or buffered glucose in the absence of penicillin, they declined in their subsequent ability to incorporate [1-¹⁴C]-glycine during very short periods, even though incorporation over long periods of up to 1 hr. at 37° appeared approximately linear. These factors led to the choice of 10 min. incubation at 20° for the measurement of mucopeptide synthesis.

Fig. 6 shows the progress of inhibition plotted as a first-order reaction. There is a short lag during which the benzylpenicillin has no effect on mucopeptide synthesis, followed by a period during which the synthetic system is inhibited strictly according to first-order kinetics. Unfortunately, the experiment could not meaningfully be extended beyond 60 min. since the control suspension also started to decrease in activity. The length of lag and the slope of the line for the progress of inhibition were very constant from experiment to experiment. The true length of the lag period is, of course, likely to be longer than 10 min., perhaps even as long as 15 min., owing to continued reaction of benzylpenicillin with the relevant sites during the period used for mucopeptide synthesis. Attempts to perform the experiment at 37° with a period of only 3 min. for mucopeptide synthesis were not successful because of the rapid apparent decay already mentioned in the initial rate of mucopeptide synthesis by the control cells. When concentrations of benzylpenicillin less than 0.05 µg./ml. were added directly to cells already suspended in the solution for studying mucopeptide synthesis, the delay in action of the antibiotic predicted on the basis of the above

experiment was discernible during subsequent incubation. At concentrations of more than 0.05 µg./ml. this delay was too short to be perceptible. This sort of delay in the action of benzylpenicillin is, of course, of quite a different order from that observed by Rogers & Jelaszewicz (1961). A possible explanation of the latter has already been given.

The important point to notice at this juncture is that irreversible fixation of benzylpenicillin and reaction of the antibiotic with the sites involved in the synthesis of mucopeptide have quite different kinetics.

Behaviour of cells in which the irreversible sites are fully saturated

Suspensions of *Staph. aureus* strain Oxford were treated at 20° with benzylpenicillin under conditions sufficient to saturate the irreversible sites studied in experiments such as those reported in Figs. 4 and 5 above. The amount of mucopeptide synthesized by such saturated cells was then judged both by the incorporation of [1-¹⁴C]glycine and by chemical estimation.

As shown in Figs. 7(a) and 7(b), after about 40 min. mucopeptide synthesis by the control cells and by those that had been pretreated with benzylpenicillin proceeded at about the same rate. During the first 40–50 min. the cells that had been pretreated with benzylpenicillin showed an acceleration in synthesis. The progress of mucopeptide synthesis was similar whether biosynthesis was measured by incorporation of radioactivity or by chemical means, and it did not matter whether an excess of benzylpenicillin was used for treatment of the cells or exactly enough to saturate them (see Fig. 5).

If one accepts the older ideas mentioned in the introduction to this paper and supposes the fixed benzylpenicillin to be that which is important in preventing growth and hence, one presumes, cell-wall synthesis, then an explanation of the present results would be that the fixed benzylpenicillin is removed during the lag period shown in Fig. 6. However, when [9-¹⁴C]benzylpenicillin was used to saturate the cells, the loss of benzylpenicillin during incubation for 1 hr. under the conditions necessary for mucopeptide synthesis was less than 10%. In other words, synthesis eventually proceeded at maximum rate in this system with at least 90% of the fixed benzylpenicillin still attached to the cells.

Rate of mucopeptide synthesis and loss of fixed penicillin

A further possible explanation of the failure of most of the fixed benzylpenicillin to affect mucopeptide biosynthesis concerns the rate of muco-

Fig. 7. Synthesis of mucopeptide by cells that had been presaturated with benzylpenicillin. (a) Measured by incorporation of $[1\text{-}^{14}\text{C}]$ glycine. A suspension of *Staph. aureus* strain Oxford was pretreated for 30 min. with either 2 $\mu\text{g.}$ (○—○) or 0.3 $\mu\text{g.}$ (●—●) of benzylpenicillin/ml. for 30 min. at 20° and then washed twice at 0° with 0.1 M-phosphate buffer, pH 7.0. After the washing, the cells were suspended at a density of 2 $\mu\text{g./ml.}$ in glucose-phosphate buffer of twice the finally required strength. This suspension was then warmed to 35° and added to an equal volume of the usual amino acid mixture containing chloramphenicol and $[1\text{-}^{14}\text{C}]$ glycine also warmed to 35° and of twice the required concentration. Incubation was continued at 35° and samples (5.0 ml.) were taken into 0.5 ml. of 50% (w/v) trichloroacetic acid. Mucopeptide was then prepared in the usual way. Suspensions of cells were kept at 20° without benzylpenicillin, washed and otherwise treated in the same way as the benzylpenicillin-treated cells to form the controls (△—△ and ▲—▲). (b) By chemical estimation. The suspension was pretreated with 2 $\mu\text{g.}$ of benzylpenicillin/ml. to give the curve shown as ○—○ and the control cells were given the same incubation and washing to give the curve ●—●. Mucopeptide biosynthesis was carried out as above and estimated chemically as described in the Methods section.

peptide synthesis by the thick suspensions (1 mg./ml.) of bacteria in the amino acid mixture. Under these conditions with *Staph. aureus* strain Oxford, a simple measurement of the time required to double the amount of mucopeptide showed that synthesis in the absence of any penicillin was proceeding at only about 20–30% of the rate in a growing culture. It could then be argued that about 90% of the benzylpenicillin remaining fixed to the cells was attached to sites that were only non-functional under the particular conditions used here for mucopeptide synthesis. The 10% lost during the lag period might then have been attached to active sites in the suspension.

Fortunately, however, when *Staph. aureus* strain Oxford was grown in a casein-hydrolysate medium of the type used by Rogers (1945) to a density equivalent to about 0.06 mg. dry wt. of cells/ml. and chloramphenicol was then added, mucopeptide synthesis went on at about the same rate as it had been occurring in the growing culture. The increase after the addition of chloramphenicol was arithmetic, of course, and not exponential. The effect of saturating the cells with benzylpenicillin was

examined under two conditions. In the first, chloramphenicol was added along with the benzylpenicillin to the culture in casein hydrolysate. In the second, no chloramphenicol was added, but only benzylpenicillin. Thus, under the second set of conditions, protein synthesis could continue during the fixing of benzylpenicillin. Sufficient $[9\text{-}^{14}\text{C}]$ -benzylpenicillin was used so that under the conditions chosen the smaller number of cells present here than in the suspensions (1 mg./ml.) was just saturated (0.1 $\mu\text{g.}$ of antibiotic/ml. was required). The bacteria were then washed twice in casein hydrolysate without thiamine and nicotinic acid and resuspended in the full casein-hydrolysate medium with 50 $\mu\text{g.}$ of chloramphenicol/ml. but with no benzylpenicillin present. Table 2 summarizes the results obtained. Somewhat more benzylpenicillin is fixed when the bacteria are in the casein-hydrolysate medium and when chloramphenicol is present (condition I) than when they are suspended in phosphate buffer, and a lot more is fixed when protein synthesis can occur during fixation because chloramphenicol is not present (condition II). Of this material about 15–17% is

Table 2. Results obtained by saturating cells with penicillin and measuring mucopeptide synthesis in casein-hydrolysate medium

Staph. aureus strain Oxford was grown to 0.06 mg. dry wt./ml. in a casein-hydrolysate medium. [9-¹⁴C]Benzylpenicillin (0.1 μ g./ml.) with (condition I) or without (condition II) chloramphenicol (50 μ g./ml.) was added and incubation at 35° continued for 15 min. At the end of this time the bacteria were deposited by centrifugation, washed with casein hydrolysate and resuspended to the same density in fresh medium containing chloramphenicol. Incubation was continued at 35° with shaking to supply aeration. Samples were taken for mucopeptide estimation by the chemical method. Samples were also taken before and after 2 hr. incubation. The amount of [9-¹⁴C]benzylpenicillin associated with the cells was measured.

Conditions during fixing of benzylpenicillin	Time for mucopeptide to double (min.)	Lag in mucopeptide synthesis (min.)	Benzylpenicillin fixed (disintegrations/min./mg.)	
			Before	After
With chloramphenicol (condition I)	35	40-50	290	240
No chloramphenicol (condition II)	38	40-50	390	224
0.1M-Phosphate buffer, pH 7.0	85-110	40-50	196	178

lost after fixation under condition I and as much as 48% after treatment under condition II. In another experiment the loss under condition II was lower, being about 30%. However, irrespective of the amount lost, the maximum rate of synthesis and the time required to attain this maximum rate were the same (Table 2). The rate of synthesis was about three times that in the amino acid mixture. Thus it seems that the carriage of 80-85% of the fixed benzylpenicillin is still compatible with a rate of synthesis of mucopeptide similar to that occurring in a growing culture. The reason for the greater amount fixed and lost when protein synthesis is going on (i.e. under condition II) has not been explained.

Creation of new sites in bacteria already saturated with benzylpenicillin

When cells were first saturated with [9-¹⁴C]-benzylpenicillin, washed, allowed to synthesize mucopeptide for 1 hr. and then treated again with the [9-¹⁴C]benzylpenicillin, equivocal results were attained but a slight, significant, gain in radioactivity appeared to result from the second treatment. To obtain clear results it was necessary first to saturate the cells with unlabelled benzylpenicillin by treating a suspension (1 mg./ml.) in 0.1M-phosphate buffer with 0.3 μ g. of unlabelled benzylpenicillin/ml. for 30 min. at 20°. The cells were then washed twice and incubated in the usual way in the four-amino acid-chloramphenicol mixture. At suitable times during incubation samples were removed and mixed with sufficient [9-¹⁴C]benzylpenicillin to give a final concentration of 0.3 μ g./ml. The mixtures were left for 15 min. at 30° and centrifuged. The cells were then washed twice with 0.1M-phosphate buffer and their radioactivity was measured. This radioactivity then indicates any

Fig. 8. Appearance of sensitive sites during formation of mucopeptide. The cells were first treated with unlabelled benzylpenicillin and thoroughly washed. They were then allowed to make mucopeptide for the times shown on the abscissa and treated with [9-¹⁴C]benzylpenicillin, washed, and the radioactivity they had fixed was measured to give the curve ●—●. In another experiment the formation of mucopeptide was measured by the incorporation of radioactivity from [1-¹⁴C]glycine into mucopeptide, with staphylococci that had first been treated with 0.3 μ g. of unlabelled benzylpenicillin/ml. The conditions for this latter part of the experiment, the results of which are plotted in the curve ○—○, were exactly the same as those shown in Fig. 7.

new sites capable of fixing benzylpenicillin that have been revealed during the incubation under conditions where mucopeptide is formed. Fig. 8 shows the results obtained, and also the results from another experiment in which the incorporation of radioactivity from [1-¹⁴C]glycine into mucopeptide was measured to indicate the progress of synthesis by bacteria saturated with unlabelled benzylpenicillin. This curve is, of course, similar to those

shown in Fig. 7(a). The rate of formation of mucopeptide becomes maximum and constant at just about the same time that the maximum number of new fixation sites is revealed. These 'new' sites will presumably be partly due to the small loss of benzylpenicillin from old sites mentioned above and partly to a true revelation of new sites. To distinguish these points of attachment of benzylpenicillin molecules from the points at which the bulk of the benzylpenicillin is fixed, they are referred to as 'sensitive' sites, and the rest are called 'irrelevant' sites.

Sensitivity of staphylococci pretreated with benzylpenicillin to freshly added antibiotic

Staphylococci were first treated with sufficient benzylpenicillin to saturate them (i.e. with 0.3 µg./ml. for 30 min. at 20°), washed thoroughly and then incubated in the four-amino acid-chloramphenicol solution with small further amounts of benzylpenicillin present. The results for inhibition of mucopeptide synthesis are shown in Table 3 together with those for staphylococci that had not been first saturated with benzylpenicillin but that had otherwise been treated in the same way. The cells already saturated with benzylpenicillin are

about two orders of magnitude more sensitive to the exogenous benzylpenicillin. Significant inhibition was obtained at about 20–40 µg./ml. with the control cells, whereas a concentration of only 0.5 µg./ml. was required with the saturated cells. Exactly similar results were obtained if the cells already saturated with benzylpenicillin were first incubated in the four-amino acid-chloramphenicol mixture for 30–40 min. before the small amounts of benzylpenicillin were added. Thus the staphylococci saturated with benzylpenicillin were still as sensitive to the antibiotic after mucopeptide synthesis had accelerated to its maximum rate.

Reaction of benzylpenicillin with the sensitive sites

The correspondence between the appearance of sensitive sites and the attainment of a maximum rate of mucopeptide synthesis shown in Fig. 7 prompted an experiment to measure the degree of saturation of these sensitive sites by the very low exogenous concentrations of benzylpenicillin required to inhibit the cells already saturated with benzylpenicillin. To do this a suspension (1 mg./ml.) of staphylococci was first treated with 0.3 µg. of unlabelled benzylpenicillin/ml. under the conditions necessary for saturating all the sites available. The bacteria were then washed twice in 0.1M-phosphate buffer, pH 7.0, and resuspended in phosphate-glucose buffer and volumes added to two series of flasks. All the flasks contained the four amino acids and chloramphenicol. In the first series different low concentrations of [9-¹⁴C]benzylpenicillin were added. In the second the same low concentrations of unlabelled benzylpenicillin were added and [1-¹⁴C]glycine was included to measure

Table 3. *Sensitivity to fresh benzylpenicillin of staphylococci that had first been saturated with the antibiotic*

Suspensions of *Staph. aureus* strain Oxford in 0.1M-phosphate buffer (1 vol.) were first incubated at 20° for 30 min. with either 1.0 µg. (A) or 0.3 µg. (B) of unlabelled benzylpenicillin/ml. They were then washed twice with 0.1M-phosphate buffer (2 vol.), resuspended in phosphate-glucose buffer and added to the four-amino acid-chloramphenicol mixture containing [1-¹⁴C]glycine and the indicated concentrations of benzylpenicillin. The control cells were treated identically except that benzylpenicillin was omitted from the pretreatment. Incubation was for 1 hr. at 35°. The maximum concentration in the final mixture of free benzylpenicillin present as a result of the first pretreatment with 1.0 µg. of the antibiotic/ml. could be not more than 1.0 µg./ml. N.E., Not examined.

Concn. of benzylpenicillin (µg./ml.)	Inhibition of incorporation of radio-activity into mucopeptide (%)			
	Control	Pretreated bacteria		
		A	B	
0.5	0	N.E.	8	17
5.0	0	N.E.	20	31
10	0–7	52	34	41
20	0–18	60	48	62
50	40–59	66	N.E.	N.E.

Table 4. *Relationship of the reaction of sensitive sites in staphylococci previously saturated with benzylpenicillin to the inhibition of mucopeptide synthesis*

This experiment was conducted as described in the text. N.E., Not examined.

Time of incubation ... Concn. of benzylpenicillin (µg./ml.)	Saturation of revealed sites (%)		Inhibition of mucopeptide biosynthesis (%)	
	30	90	30	90
0.5	2	4	14	15
5.0	17	27	30	30
10	26	38	35	44
20	45	52	56	64
50	82	69	N.E.	66

mucopeptide synthesis. Samples were taken at 30 min. and 90 min. from both series of flasks during incubation with shaking. The organisms from the first series were washed twice and the radioactivity remaining from [9-¹⁴C]benzylpenicillin was estimated to indicate the saturation of the sensitive sites. Mucopeptide was prepared from the organism in the second series of flasks and the radioactivity contained by the samples estimated to indicate mucopeptide synthesis. From these measurements the extent of blocking of the sensitive sites and the degree of inhibition of mucopeptide synthesis could be calculated.

The agreement between the saturation of the sensitive sites and the inhibition of mucopeptide biosynthesis is by no means perfect (Table 4). Nevertheless it is sufficiently impressive to suggest that these sites should not be combined with benzylpenicillin molecules if mucopeptide synthesis is to take place.

DISCUSSION

The inhibition by penicillin of mucopeptide synthesis by whole staphylococci is shown to be fully reversible after a lag of 40 min. or so during incubation in a medium containing an excess of chloramphenicol. A high proportion of the 170 molecules of benzylpenicillin that remain firmly fixed to a staphylococcus seem not to be combined with sites concerned with mucopeptide biosynthesis. Alternatively, if they are so combined they do not inhibit the functioning of these sites, since mucopeptide synthesis goes on at the same rate whether or not 80–90% of the fixed benzylpenicillin is present. Moreover, the kinetics of the reaction between benzylpenicillin and most of the fixation sites in the cell are different from those for the reaction of the antibiotic with sensitive sites involved in mucopeptide biosynthesis. Far from being involved in inhibiting mucopeptide biosynthesis, the presence of most of the irreversibly fixed molecules seems to make the bacteria very much more sensitive to additional benzylpenicillin. Significant inhibition of mucopeptide biosynthesis when the cells had already been saturated with benzylpenicillin was detectable with a concentration of 1.5 μ M-benzylpenicillin, this being about 50–100 times less than that required for the same effect on normal cells not carrying benzylpenicillin. In other words, the fixation of most of the benzylpenicillin to the cells appears to be a primitive detoxication mechanism.

This increased sensitivity of cells already carrying a full load of fixed benzylpenicillin may possibly be explained simply from the kinetics and characteristics of fixation process compared with those of the combination with the sensitive sites involved in

mucopeptide synthesis. When the two reactions are occurring simultaneously, both of which have a common reactant (benzylpenicillin) in limited supply, competition by the 'irreversible' sites for benzylpenicillin molecules will decrease as the reaction proceeds since these react by a second-order process. The initial lag in the action of benzylpenicillin on the sensitive sites may be because almost all the available benzylpenicillin molecules may initially react with the irrelevant fixation sites. The numbers of molecules of benzylpenicillin available for both reactions may be limited by the rates of diffusion to the reactive sites.

The exact nature of the sensitive sites involved in mucopeptide synthesis with which benzylpenicillin combines is not clear. Not more than about 30–40 molecules of benzylpenicillin/cell seem to be involved. The appearance of these sites occurs during the period during which mucopeptide synthesis by cells already loaded with fixed benzylpenicillin accelerates. When mucopeptide synthesis reaches a maximum rate, the number of new sites is maximal and remains steady. The degree of substitution of these new sites with benzylpenicillin molecules approximately matches the degree of inhibition of mucopeptide synthesis. They appear during incubation of the benzylpenicillin-loaded cells in a mixture of amino acids that contains an excess of chloramphenicol. It is therefore not likely that they are associated with proteins formed during the incubation, since chloramphenicol suppresses protein formation. In cells not loaded with benzylpenicillin (i.e. the control cells in the experiments described), these sites either do not appear or are not functional in mucopeptide biosynthesis. If functional, they do not limit mucopeptide formation. Otherwise mucopeptide synthesis by untreated cells would also accelerate with time during incubation, whereas this has never been seen to happen.

The characteristics of the material bearing the sensitive sites are then likely to be that of a non-protein substance, located somewhere in the surface layers of the cell, and, since it is now known that penicillin inhibits the late cross-linking transpeptidation reaction (Wise & Park, 1965; Tipper & Strominger, 1965; Izaki *et al.* 1966) in mucopeptide biosynthesis, it is likely to be in some way concerned with such a reaction. The possibility exists that the material is incompletely formed mucopeptide or mucopeptide precursors rather than enzymic proteins involved in forming the cross-links. If penicillin combined with the ϵ -lysine groups in some form of incomplete mucopeptide or the free amino group of polyglycine chains, it might be expected to block completion of the polymer by the enzymes involved in the cross-linking reactions. Penicillin has been shown to combine with the ϵ -amino groups

in polylysine (Schneider & de Weck, 1965) and in proteins (Batchelor, Dewdney & Gazzard, 1965), forming penicilloyl compounds. Such a type of reaction would explain both the inhibition of the cross-linking reaction and the present results. It would not, however, explain the strong inhibition of the D-alanine carboxypeptidase in extracts prepared from *E. coli* strain B (Izaki *et al.* 1966). However, there are a number of points concerning the cell-free systems from *E. coli* that do not seem to be consistent with results obtained with staphylococci. For example, inhibition of transpeptidation by penicillin is irreversible, and the incomplete mucopeptide formed is water-soluble in the *E. coli* system. The latter result agrees with my own finding, with staphylococci, that both net synthesis and incorporation of amino acids into mucopeptide prepared by the Park & Hancock (1960) type of method are strongly inhibited by low concentrations of benzylpenicillin. It does not, however, seem to agree with the observed accumulation of incomplete but apparently insoluble mucopeptide in the preparations from walls of staphylococci examined by Wise & Park (1965) and Tipper & Strominger (1965). Again, in staphylococci the inhibition both of growth and mucopeptide synthesis is fully reversible after the action of benzylpenicillin. Moreover, the reversal of mucopeptide synthesis occurs in a situation where no more protein is formed.

Such a hypothesis for the action of penicillin leads us back to two primary observations that have been made on the action of the antibiotic on staphylococci. These are: (i) that small uridine precursors of mucopeptide accumulate; (ii) that incomplete mucopeptide of the type shown in Fig. 1 can also accumulate (Wise & Park, 1965; Tipper & Strominger, 1965) when mucopeptide biosynthesis by staphylococci is inhibited by penicillin. Even if penicillin can combine with the ϵ -amino group of lysine in the uridine precursor, the type of precursor could still only be explained if the blocking of amino groups also leads to some sort of inhibition of further glycoside-bond formation between *N*-acetylglucosamine and *N*-acetylmuramic acid or, more likely, if the formation of the glycoside bond proceeds very much more efficiently after formation of the peptide bond of the cross-link. Combination of penicillin with the ϵ -amino group of lysine in the uridine precursors of mucopeptide themselves cannot be a very efficient process, if it does occur, since large amounts of penicillin fail to inhibit the incorporation by cell-free systems of these together with glycine into incomplete mucopeptide (Chatterjee & Park, 1964; Meadow, Anderson & Strominger, 1964). If, then, penicilloyl compounds are formed between penicillin and the primary amino group of glycine in incomplete mucopeptide the appearance of the sensitive sites in cells pre-

saturated with penicillin, when the bacteria are incubated in the presence of chloramphenicol, would be explained. In the absence of free penicillin the formation of new mucopeptide can still go on, but it could only be accepted by the existing mucopeptide where amino groups are free to react, which could only happen by hydrolysis of the penicilloyl bond. This would then suppose a positive rate of hydrolysis of the amino group-penicilloyl compounds during the lag phase in mucopeptide synthesis shown by penicillin-loaded cells, and indeed a very small loss of 'fixed' penicillin has been observed. This hypothesis for the action of penicillin would also explain the observed failure by the cell to attach the fragments of mucopeptide ribitol teichoic acid to the remainder of the mucopeptide (Rogers & Garrett, 1965). If by the formation of the peptide cross-bridges the efficiency of the formation of glycoside bonds is affected, the fragment with teichoic acid might not become properly attached to the rest of the mucopeptide. The failure (Duerksen, 1964) to find fixed penicillin in purified wall preparations after treatment of the organisms with the antibiotic is understandable if the penicilloyl bonds are hydrolysed rather readily and if at least part of the reaction occurs before the formation of the glycoside bonds necessary for complete insoluble mucopeptide. The small amount of incomplete material may stay associated with the membranes rather than the walls of the bacteria.

It is recognized that the above hypothesis is directly opposed to other current hypotheses (Wise & Park, 1965; Tipper & Strominger, 1965; Collins & Richmond, 1962), all of which suppose the combination of penicillin with enzymes concerned with mucopeptide synthesis, but so far it is not apparent that available evidence makes either type of hypothesis the more likely.

The significance of the correlation reported earlier (Eagle, 1954*a,b*; Rowley *et al.* 1950) between sensitivity and the total binding of penicillin by representatives of various species and genera of bacteria is not always easy to analyse. Many factors may limit the rate and extent of reaction of penicillin with all the binding sites in the cell, and some of these factors will be shared by both the specific sensitive sites and the irrelevant ones, whereas others may not. For example, if an organism produces penicillinase, the organism will be both insensitive to penicillin and fail to bind the antibiotic. This would explain both the insensitivity of *E. coli* K-12 and its inability to bind penicillin, a point made by Eagle (1954*a*) at the time. If an organism is relatively impermeable or the binding sites are relatively inaccessible to penicillin, this again might limit both characteristics. It appears possible that such factors would explain the correlation observed for the strains of *Streptococcus*

pyogenes, of *Diplococcus pneumoniae*, of *Staph. aureus* and of *Strep. faecalis*. The whole organisms bound 1.81–3.28, 1.44–2.0, 0.97–1.09 and 0.046–0.086 μg . respectively of penicillin/g. when growing cultures were treated with 0.01 μg . of the antibiotic/ml. The differences between the strains became greater when lower concentrations of antibiotic were used for treatment. When ultrasonically disrupted materials from these same four bacteria were treated with the same concentration of antibiotic they bound 0.93–1.15, 0.7–1.49, 1.02–1.69 and 0.16–0.23 μg . respectively of penicillin/g. The minimum lethal doses of penicillin for these organisms were 0.006, 0.018, 0.024 and 2.0 μg /g. Thus the apparent correlation between sensitivity and binding disappeared altogether when disrupted materials from the first three strains were used, and became less satisfactory for the fourth, *Strep. faecalis*.

When the four species of micro-organisms referred to in the last paragraph were made resistant to penicillin by selection in the laboratory (Eagle, 1954b), the resistant mutants sometimes bound a little more of the antibiotic or sometimes a little less and no correlation existed. Moreover, as the mutants were kept in the laboratory, resistance to penicillin and binding of the antibiotic changed independently. Thus one is forced to conclude that such correlation as existed between the total binding of penicillin and the sensitivity of the bacteria to the antibiotic may well have been due to causes other than variation in the number of specific important reactive sites. The present work amply confirms such a conclusion. In justice to the earlier work, I should perhaps finish by a quotation from one of the earliest papers: 'It is entirely possible that the observed uptake is due to a mechanism unconnected with the antibiotic activity of penicillin . . . ' (Maas & Johnson, 1949).

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