REVIEW ARTICLE

THE MODE OF ACTION OF PENICILLIN

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Although penicillin has been used successfully in clinical medicine for some 20 years its precise mode of action has until recently been ill understood. Now, progress can be reported.

Previous reviews on its mode of action have been published by Rinderknecht (1946), Pratt and Dufrenoy (1947) and Eagle and Saz (1955), and Stenlake (1959) has recently summarised the actions of various antibiotics. Because one of the main effects of penicillin appears to be concerned with an inhibition of bacterial cell wall synthesis, the greater part of this review will deal with cell wall structure and the effect thereon of the antibiotic.

After this review was completed, a review on the mucopeptide components of bacterial cell walls was published by Work (1961) who described briefly the effect of penicillin on cell wall synthesis.

Morphological Changes Induced by Penicillin

Gardner (1940) reported that, in dilute solutions, penicillin induced a distinct lengthening in all the rod-shaped bacteria which were sensitive to the antibiotic. In a later report, Gardner (1945) extended his work to a microscopical examination of the effect of penicillin on the spores and vegetative cells of bacilli, and found that even the weakest inhibitory dose of penicillin attacked the organism in the early stages of germination. It was also shown that lytic changes in vegetative cells of *Bacillus anthracis* were less pronounced in a strong than in a weak penicillin solution.

Thomas and Levine (1945) demonstrated that penicillin in inhibitory but not completely bacteriostatic concentrations induced bizarre involution forms in Gram-negative intestinal bacteria growing in liquid or solid media. Fisher (1946) showed that the *in vitro* activity of penicillin on staphylococci caused enlargement of the bacterial cells followed by lysis. Similar effects were observed to a lesser degree on cultures of β-haemolytic streptococci and pneumococci. Fisher also made the interesting observation that the group A streptococcus was not killed in the same manner, since there was no evidence of debris to suggest that many of the bacteria had been lysed, although cultures were almost sterile after 10 hr. and completely so after 24 hr.

Duguid (1946) showed that low concentrations of penicillin induced giant forms in *Escherichia coli* and Hughes, Kramer and Fleming (1946) described the morphological changes induced by penicillin in *Proteus vulgaris*; these included (*a*) elongation up to 200 μ in length, and (*b*) production of single or multiple swellings on the rods. Often, completely spherical, actively motile forms of 6 μ or less could be observed on microscopical examination.

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The changes occurred most rapidly at 37°, and with higher concentrations of penicillin.

Later, Pulvertaft (1952) showed that at critical concentrations, penicillin, streptomycin, aureomycin, terramycin and chloramphenicol induced bacteriolysis of *E. coli* but stated that penicillin alone permitted bacterial enlargement at all concentrations followed by lysis. Pulvertaft (1952) also observed that if growth occurred after the pencillin had been neutralised, the first organisms formed were always giant forms. This is in the pattern of events found by Parker and Marsh (1946) and Eagle and Musselman (1949), who showed that the effect of the antibiotic persisted for some time after its removal.

Hughes (1955a, b; 1956) investigated the effect of penicillin on the morphology of *P. vulgaris* and demonstrated that even when the culture was derived from a single cell, variations were apparent between the individual cells; for example, some developed into long forms, some lysed, and some were relatively unaffected.

The morphological changes induced in bacterial cells by antibiotics have also been studied by Kamijo (1953a, b; 1954a, b, c, d) and by Takahashi, Sukeyuki and Kamijo (1957). The antibiotics used were penicillin, streptomycin, chloramphenicol, terramycin and erythromycin.

Liska (1959) has recently reported that penicillin causes swelling and elongation of *Streptococcus lactis*, *Streptococcus thermophilus* and *Leuconostoc dextranicum*.

Penicillin and Bacterial Growth

It was early realised that penicillin was active against dividing bacteria. Thus Hobby, Meyer and Chaffee (1942a) reported that, depending on the experimental conditions, penicillin acted as a bacteriostatic or bactericidal agent, and that it appeared to be effective only when active bacterial multiplication was taking place. This was confirmed by Chain and Duthie (1945), Hobby and others, (1942a), Lee, Foley and Epstein (1944), and Bigger (1944) offered evidence to show that penicillin exerted no bactericidal action in nutrient broth at temperatures sufficiently low to prevent the bacteria from multiplying.

Todd (1945a) showed that the most rapid bacteriolysis induced by penicillin occurred with organisms at the maximal rate of multiplication, and Knox (1945) found that young cells were particularly sensitive to the antibiotic, which was confirmed by Pratt and Dufrenoy (1947). Conditions which increased the rate of growth of haemolytic streptococci were found to increase the rate at which penicillin acted on these bacteria (Hobby and Dawson, 1944a, b), and Hahn and Ciak (1957) found that penicillin-induced lysis, as determined by "protoplast" formation, occurred only in an invironment capable of supporting bacterial growth. Pandalai and George (1947) suggested that penicillin appeared to be primarily bacteriostatic, inhibiting the growth of the organism by preventing cell division and multiplication. Duguid (1946) stated that only actively metabolising and growing cells were susceptible to penicillin and suggested that in low concentrations the antibiotic interfered with the formation of the outer

supporting wall; he also stated, however, that in high concentrations the antibiotic must act differently.

Some disagreement with the above was expressed by Schwartzman (1945), who found that the susceptibility of certain strains of *E. coli* and *Salmonella sp.* was significantly greater in synthetic medium than in meat infusion broth. Differences in penicillin activity were not caused by changes in the rate of growth in the media used.

Although penicillin is generally thought to be effective only against actively multiplying cells, it is by no means correct to state that it is without effect on resting organisms, for Garrod (1945) found penicillin to have a marked bactericidal effect on staphylococci at 10° and even at 4°, although no active cell division had occurred. Eriksen (1946) made a similar observation on the anthrax bacillus at 4°.

Garrod (1945) also showed that disinfection of *Staph. aureus* (Oxford strain H) was more rapid at 42° than at 37°, although growth at the higher temperature had ceased. With their strain of staphylococcus, however, Lee and Foley (1945) found that although the organism grew more slowly at 42° than at 37°, there was no significant difference between the rates of killing by penicillin at the two temperatures. They further found that at 50–60° growth ceased altogether and the bacteria began to die through the effect of heat, but even at these high temperatures the rate of reduction of viable cells in tubes containing penicillin was higher than that in control tubes.

Lee and others (1944), showed that high concentrations of penicillin (88 units/ml.) accelerated the death rate of staphylococci suspended in saline, and a similar result was obtained by Garrod (1945) with Ringer's solution. It must be pointed out here that Chain, Florey, Abraham and Heatley (1949) have drawn attention to the fact that Lee and others (1944) used a crude preparation (150 units/mg.) and that it was possible that the bactericidal effect demonstrated in saline was directly or indirectly caused by an impurity. It has been mentioned earlier, however, that Lee and others (1944) found that staphylococci in broth at 14° were unaffected by penicillin. If this had contained impurities, a similar bactericidal effect would have been expected to occur here.

Gunnison, Kunischige, Coleman and Jawetz (1955) investigated the effect of antibiotics *in vitro* on bacteria which were not actively multiplying, and showed that penicillin had some bactericidal effect at 37° on washed suspensions of *Staph. aureus* and *E. coli*, although high concentrations of antibiotic (500 units/ml.) were needed.

Penicillin and Lysis

Hobby, Meyer and Chaffee (1942b) found that increasing the concentration of penicillin above a certain level did not significantly influence its rate of bactericidal effect. This was confirmed by Lee and others (1944). Eagle (1948) also showed that the rate at which organisms were killed by penicillin rose to a maximum as the concentration of the drug increased, but found that with some strains of *Staph. aureus*, *Streptococcus faecalis*, and Group B β-haemolytic streptococci the organisms were killed

much more slowly when the penicillin concentration increased beyond a certain level. Kirby (1945) had earlier noticed that many more viable cells could be recovered from a staphylococcal suspension treated with 100 units/ml of penicillin than from one treated with 0·1 unit/ml., and considered this phenomenon to be a consequence of the greater retardation of the initial growth rate brought about by the larger amount of the antibiotic. Similarly, lysis of vegetative cells and spores of *B. anthracis* was more complete in concentrations near the inhibitory level of, for example, 1 unit/ml. than in higher concentrations of, for example, 100 units/ml. (Gardner 1945). Commenting on these results, Chain and others (1949) have stated: "It would appear . . . that penicillin in high concentration may have a secondary effect in suppressing some phase of the activity of certain organisms which is essential for the bactericidal action of the drug."

Eagle (1953) suggested that the accumulation of a toxic intermediate was caused by low concentrations of penicillin, whilst higher concentrations produced a secondary effect which reduced the production, or increased the destruction, of this substance.

Todd (1945b) suggested that bacteria may first be killed by penicillin and then undergo lysis through the action of autolytic enzymes; Abraham and Duthie (1946) pointed out that most of the organisms in a culture may be killed before lysis begins, which was confirmed by Gale and Taylor (1947) and more recently by Hurwitz, Reiner and Landau (1958) who found that cells of *E. coli* showed a loss of viability well before osmotic fragility became apparent.

Bonét-Maury and Pérault (1945) found that when a suspension of staphylococci lysed with small concentrations of penicillin was incubated for a further period of time, survivors which had withstood the initial bactericidal effect of the antibiotic were able to divide. This was followed, however, by a secondary lysis which occurred after 24 to 48 hr. incubation. These results have been confirmed by Abraham and Duthie (1946).

Penicillin and Amino-acid Assimilation

Gram-positive organisms, unlike several gram-negative bacteria such as *E. coli* and *Klebsiella aerogenes*, are unable to synthesise amino-acids. Certain Gram-positive bacteria, for example, staphylococci or streptococci, have acquired a mechanism for the concentration of amino-acids which compensates for this loss of synthetic ability (Gale, 1952). They possess a cell wall or membrane which enables them to actively assimilate certain amino-acids and concentrate them in the cell before metabolism or concentration into protein. Basic amino-acids, like lysine, were found by Gale (1947) to be able to diffuse through the cell wall of these Gram-positive bacteria, whilst acidic amino-acids, such as glutamic acid and aspartic acid, were unable to penetrate the wall unless an exogenous energy source was also available.

It has recently been found that Gram-negative bacteria can also effect high concentration gradients of amino-acids (Cohen and Rickenberg, 1956); there is, however, a rapid equilibration between the internal and

external medium. The technique originally employed by Gale (1947) involved extensive washing of the cells before the estimation of the internal amino-acids, so that, as pointed out by Gale (1959), the internal amino-acids are rapidly lost to the external medium.

Gale and Taylor (1947) found that, shortly after its addition to the medium, penicillin prevented growing cultures of *Staph. aureus* from accumulating glutamic acid. It had no significant effect upon the accumulation of lysine within the cells. Penicillin, 50 units/ml., inhibited the assimilation of glutamic acid by washed suspensions by only 10 per cent. The results indicated that the prevention of glutamic acid assimilation preceded the failure of respiration and the onset of general lysis, and appeared to take place with or before loss of viability. It was suggested that penicillin either combined with, or produced a reorganisation of, the cell wall such that the assimilatory mechanism was blocked.

Hancock (1958) showed that the addition of M sucrose or M sodium chloride (but not glycerol) to the medium protected the cells from the inhibition of amino-acid transport by penicillin; the inhibitions which occurred in the absence of stabilising agent were thus secondary effects (Gale, 1960).

Schwartzman (1946) showed that the resistance of Gram-negative organisms to penicillin was increased by the presence of glutamic and aspartic acids, and Wyss (1951) suggested that Gram-negative bacteria were resistant to the antibiotic because they were able to synthesise their own supply of amino-acids. It was also suggested that Gram-positive bacteria became resistant to penicillin because mutants recovered the ability to synthesise their own amino-acids.

Bondi, Kornblum, and De St. Phalle (1954) investigated the amino-acid requirements of penicillinase-producing (PP) and non penicillinase-producing (NPP) strains of *Micrococcus pyogenes*. The only major difference was found to be an impairment of the ability of NPP strains to synthesise all the amino-acids required for growth. These results are in contrast to those of Gale and Rodwell (1949) who found that artificially-resistant strains showed marked differences in their amino-acid requirements from the parent strains. Cugurra and Savora (1958), however, found that a strain of *Staph. aureus* and the same strain after being made resistant to penicillin showed the same amino-acid composition.

Hotchkiss (1950) suggested that penicillin interfered with the bacterial synthesis of amino-acids, and Simmonds and Fruton (1950) stated that the bacteriostatic action of penicillin was due to interference in the incorporation of glycine into peptide.

Gale (1958, 1959) and Gale and Folkes (1953a, b, c) have shown that penicillin has an effect on protein synthesis only in concentrations much higher than those required to inhibit growth or cell wall synthesis. Hancock and Park (1958) have been able to confirm directly that penicillin inhibits the incorporation of amino-acids into the peptide portion of the wall substance but not into the protein fraction of the cells.

Mandelstam and Rogers (1959) have also found that the incorporation of glutamic acid, glycine and alanine could take place directly into the

cell wall substance of staphylococci, that this process could occur in the absence of protein synthesis, and that it was inhibited by penicillin, but not by chloroamphenicol.

The Uptake of Penicillin by Bacteria

Cooper and Rowley (1949) found that radioactive penicillin was taken up by bacteria in amounts which increased with penicillin concentration in the external environment, and showed that there was a direct relation between the sensitivity of an organism and the amount of penicillin attached to it. This uptake was greatly increased when growth occurred in the presence of penicillin, which was confirmed by Maas and Johnson (1949a). Eagle (1953), however, was unable to confirm this, and found that cell-free bacterial extracts were able to bind penicillin, and to approximately the same degree per unit weight as intact cells. Maas and Johnson (1949b) showed that yeast cells did not bind penicillin, nor did the antibiotic penetrate the cell wall.

These workers (1949a, b) suggested that the antibiotic was bound by a component which was present in the cells in extremely small amounts. This trace component was termed the penicillin-binding component (PBC) by Cooper (1956).

By shaking cells of *Staph. aureus* with small glass beads, Cooper, Rowley and Dawson (1949) obtained a cell wall fraction and a cytoplasmic fraction, and showed that the radioactive penicillin was concentrated in the cytoplasm of the cell of this organism.

PBC was found to occur in penicillin-sensitive strains, but resistant strains (previously selected as being non penicillinase-producing strains) were either without PBC or sheltered it from penicillin at low concentrations. This was not the experience of Maas and Johnson (1949a) but by increasing the penicillin concentration to equally effective (LD99·9) levels, Eagle (1954) found that the amount bound by naturally-occurring resistant strains was relatively constant despite wide variations in their sensitivity to penicillin.

Cooper (1955) found that penicillin was bound to a lipid-containing fraction close to the cell wall. Mitchell and Moyle (1951; 1956) showed that the cytoplasmic membrane was a complex lipoprotein, and it thus seems likely that the particles which bound penicillin were originally cytoplasmic membrane.

Maas and Johnson (1949a, b) found that the bulk of the penicillin was not excreted when renewed growth and multiplication occurred, but remained in the daughter cells. This could explain the reports (Parke and Marsh, 1946; Eagle and Musselman, 1949) that, after transfer to penicillin-free medium, the first cells formed were always abnormal.

Cell Wall Structure and Penicillin Action

Before present-day knowledge of the mode of action of penicillin is reviewed, it is necessary to describe in some detail the chemical constitution of the cell wall.

Bacteria possess a rigid cell wall external to the cytoplasmic (cell, protoplasmic) membrane and cytoplasm. This wall is responsible for the shape and morphological integrity of the bacterial cell.

Dawson (1949) showed that it was possible to isolate cell walls by shaking bacteria with small glass beads in a sonic oscillator. This method can also, of course, be used for studying intracellular enzymes (Hugo, 1954).

Salton and Horne (1951) used this method to prepare cell walls of *E. coli*, *Str. faecalis*, and *Salmonella pullorum*. By measuring the ultraviolet absorption spectra of walls of the first two organisms, they were able to show that only traces of nucleic acid or purine- or pyrimidine-containing compounds were present in pure wall preparations. In a short communication, Salton (1952a) showed that the cell walls of Gram-negative bacteria contained a far higher lipid content and a more complete range of amino-acids than those from Gram-positive organisms. Salton (1952b) subsequently showed that the cell wall of *Str. faecalis* was essentially a mucopolysaccharide. The sugar components of the polysaccharide were identified as glucose, galactose and rhamnose. Pentose was absent, which substantiated the earlier finding (Salton and Horne, 1951) that no significant amounts of nucleic acid were present in the cell wall. A hexosamine was identified as glucosamine, and the predominating amino-acids were found to be alanine, glutamic acid and lysine. It was later shown that the walls of Gram-negative bacteria contained far less hexosamine than Gram-positive cell walls (Salton, 1953).

Cummins and Harris (1955; 1956) prepared cell wall suspensions of various Gram-positive species by Mickle disruption, and found that a very high proportion of the amino-acid moiety of the cell wall could in each case be accounted for in terms of three or four of glycine, alanine, lysine, glutamic acid, aspartic acid and diaminopimelic acid (DAP). The last-named was first detected in the cell walls of various bacteria by Work (1951).

Work and Dewey (1953) made a systematic investigation of the distribution of DAP among micro-organisms, and showed it to be present in nearly all the bacteria examined. The most widely distributed form was found to be the mesoform (Hoare and Work, 1957) which was present in, for example, the cell walls of *E. coli* and *Rhodospirillum rubrum* (Salton, 1957), although the L-form may also be found, for example, in the-cell walls of *Clostridium welchii* (Salton, 1957).

Cummins and Harris (1956) pointed out that the cell walls of the Gram-positive bacteria which they examined contained either DAP or lysine as a major component but not both in similar quantities, which suggested that they had similar structural functions. DAP could be decarboxylated to lysine; it might thus have been expected that the DAP decarboxylase would be found in those cases in which lysine and not DAP was a major cell wall component, but no such simple relationship existed. Work (1959) showed that DAP was decarboxylated to lysine, and that DAP was a major, but not the only, source of lysine in *E. coli*.

A hitherto unknown hexosamine, first found in bacterial spores (Strange and Powell, 1954; Strange and Dark, 1956) was provisionally characterised

as 3-O-α-carboxyethylhexosamine by Strange (1956) and termed "muramic acid". This formula was confirmed by Kent (1957).

Glucosamine and muramic acid have been found to be universal constituents of cell walls of Gram-positive bacteria (Cummins, 1956), and have also been shown to be present in the walls of several Gram-negative organisms, for example, *E. coli* and *Salmonella gallinarum* (Salton, 1957). Galactosamine may sometimes be present, as in *Clostridium welchii* (Salton and Ghuysen, 1957).

Work (1957) has emphasised that the walls of Gram-positive bacteria possess a common basal structure in which alanine; glutamic acid, muramic acid and glucosamine, and frequently DAP, appear as monomeric building blocks.

TABLE I

THE CHEMICAL COMPOSITION OF BACTERIAL CELL WALLS

Constituent	Cell walls of	
	Gram-positive bacteria	Gram-negative bacteria
Lipid	Low, about 2 per cent	High, about 20 per cent
Amino-sugar	Usually high, about 15 per cent or more	Usually low, about 2–4 per cent
Polysaccharide	Usually higher in Gram-positive organisms	
Amino-acids	4–5 main ones, only	Almost a complete range
DAP	Found in all bacterial cell walls so far tested, except staphylococci and related species.	

Reviews concerning bacterial cell walls have been published by Salton (1956; 1959), Cummins (1956), Work (1957) and Zilliken (1960). Work (1961) has recently reviewed the literature pertaining to the chemistry of the mucopeptide components of cell walls.

A summary of the chemical constitution of the cell walls of Gram-positive and Gram-negative organisms is given in Table I.

Park and Johnson (1949) described the uptake of labile phosphate in penicillin-treated *Staphylococcus aureus*, and Park (1952a, b, c) succeeded in isolating three uridine nucleotides which accumulated. These contained uridine-5′-pyrophosphate linked to an unidentified *N*-acetylamino sugar and (i) a peptide of D-glutamic acid, L-lysine and alanine in ratio 1 : 1 : 3 (Fig. 1), or (ii) a peptide of L-alanine or (iii) no peptide.

These nucleotides were found to account for a considerable fraction of the total cellular phosphate. Hotchkiss (1950) suggested that penicillin interfered with the bacterial synthesis of protein from amino-acids, the process being blocked at such a point that peptide intermediates accumulated instead.

The amino-sugar was later identified as muramic acid and Park and Strominger (1957) showed it to be present in the nucleotide which accumulated in *Staph. aureus* treated with penicillin, and that the ratio of amino sugar: D-glutamic acid: L-lysine: alanine in the cell walls of this organism was 1 : 1 : 1 : 3, which was the same as that in the nucleotide. This suggested (Park and Strominger, 1957; Strominger, Park and Thompson, 1959) that the uridine-5′-pyrophosphate *N*-acetylmuramic acid peptide was a precursor of the bacterial cell wall, and that the mechanism of action and selective toxicity of penicillin were related to the inhibition of biosynthesis of the bacterial cell wall.

Strominger (1957a, b) has suggested that the inhibition of ribonucleic acid (RNA) synthesis by penicillin was due to the side-tracking and trapping of uridine by the Park nucleotides.

Strominger and Threnn (1959) showed that the alanine of the peptide portion of the nucleotide, and of the cell wall, was composed of 1/3 L-isomer and 2/3 D-isomer. Strominger and Ito (1959) inferred that, since incomplete peptides were also induced, the peptide portion was synthesised by stepwise addition of amino-acids, and succeeded in separating each of the enzymes catalysing the stepwise addition of (1) L-alanine, (2) D-glutamic acid, (3) L-lysine, and (4) D-alanine-D-alanine. Each of these reactions required adenine triphosphate and a divalent cation.

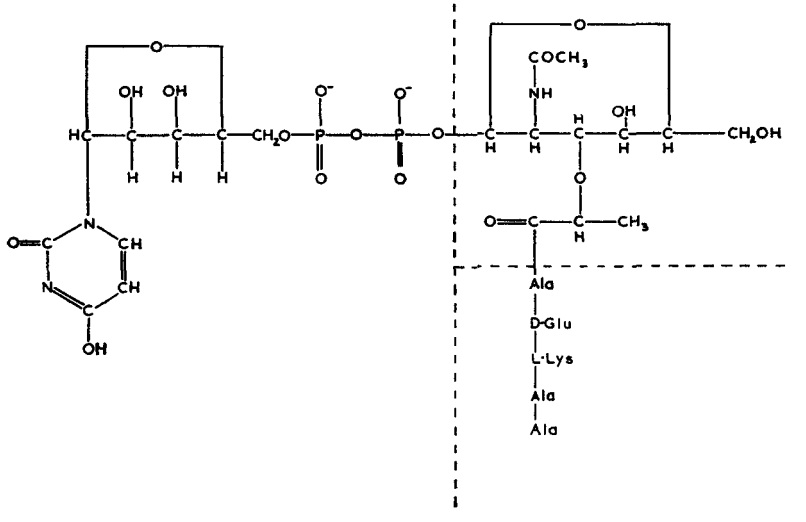

FIG. 1. 5′-Uridine pyrophosphate *N*-acetylmuramic acid peptide (Park nucleotide).

Nathensen and Strominger (1959) showed that pencillin inhibited DAP incorporation and cell wall synthesis in *E. coli* in a manner analogous to the inhibition of lysine incorporation and cell wall synthesis in *Staph. aureus*.

It has previously been stated that Cooper (1955) found that penicillin was bound at the cytoplasmic membrane. Thus, a hypothetical transglycosidase is strategically located to transfer the *N*-acetylamino sugar peptide from uridine pyrophosphate, which is inside the membrane, to an acceptor (cell wall site) outside the membrane (Park and Strominger, 1957).

Other antibacterial agents can also induce nucleotide accumulation. Thus, Gale and Folkes (1953b, c) found that bacitracin had essentially the same effects on *Staph. aureus* as penicillin, and Park (1958) reported that, in addition to penicillin, cycloserine, glycine and bacitracin caused marked accumulation of uridine 5′-pyrophosphate *N*-acetylamino sugar

derivatives in *Staph. aureus*. Chlortetracycline in high concentrations had a similar effect; in low concentrations, however, it inhibited protein synthesis. Accumulation of these nucleotides has also been shown by Abraham (cited in Park, 1958) for bacitracin, and by Strominger, Threnn and Scott (1959) for novobiocin, cycloserine and gentian violet.

Gentian violet was found (Strominger, Threnn and Nathensen, 1958) to produce a block at an earlier point in the metabolic sequence of biosynthesis of the cell wall than penicillin, inducing the accumulation of uridine nucleotides not containing amino-acids. Cytidine nucleotides have also been found to accumulate in a strain of *Staph. aureus* inhibited by this substance (Armstrong, Baddiley, Buchanan and Carss, 1958; Strominger, 1959).

Strominger, Scott and Threnn (1959) found that a DAP-requiring mutant of *E. coli* contained a high steady state concentration of a uridine nucleotide in which the peptide portion was represented as L-alanine-D-glutamic acid-meso-DAP-D-alanine-D-alanine. This compound was the analogue of (I). When the *E. coli* mutant was deprived of DAP, the DAP-containing nucleotide disappeared, and a uridine nucleotide identical to one accumulating in *Staph. aureus* deprived of lysine accumulated. The peptide portion of this was represented as L-alanine-D-glutamic acid only.

After Weibull's discovery (1953a, b) that lysozyme-induced protoplasts of *Bacillus megaterium* could be stabilised with 0·1–0·2M sucrose, came reports of "protoplast" formation induced by penicillin in *P. vulgaris* (Liebermeister and Kellenberger, 1956) in *E. coli* and *Salmonella typhimurium* (Lederberg, 1956; 1957), and in *E. coli* (Hahn and Ciak, 1957). These results were confirmed by McQuillen (1958a), and Hugo (1958) showed that the method was applicable to a wide range of Gram-negative bacteria.

Gebicki and James (1958; 1960) used this method to obtain spheres of *Aerobacter aerogenes* and Lark (1958a, b) showed that penicillin induced "globular forms" and "crescents" in *Alcaligenes faecalis*. Sphere formation has also been induced by penicillin in *Xanthomonas phaseoli* (Nozzolillo and Hochster, 1959).

Salton and Shafa (1958) carried out a chemical analysis of the "walls" of penicillin-induced spheres of *Salmonella gallinarum* and *Vibrio metchnikovii*, and showed them to contain the same amounts of lipid and polysaccharide (determined as reducing sugar) as did cell walls. On the other hand, there was a 30–50 per cent decrease of hexosamine and DAP in the sphere "walls" and McQuillen (1958a) found that penicillin-induced spheres of *E. coli* contained much less DAP in their trichloroacetic acid-precipitable fraction.

The use of the word protoplast for the spherical forms induced by penicillin was criticised by Brenner and others (1958) on the grounds that they probably had cell wall constituents attached to them (Salton and Shafa, 1958). Hurwitz and others (1958) have suggested that the term spheroplast be used to differentiate these spheres from the round forms (protoplasts) induced in Gram-positive bacteria by lysozyme. This term will be used hereafter.

Lark and Lark (1959) showed that the phenol-insoluble fraction of *Alcaligenes faecalis*, in which alanine, glutamic acid, lysine and DAP were present, was responsible for the rigidity of the cell wall of this organism. Weidel, Frank and Martin (1960) showed the cell wall of *E. coli* strain B to be composed of three layers (as had earlier been proposed

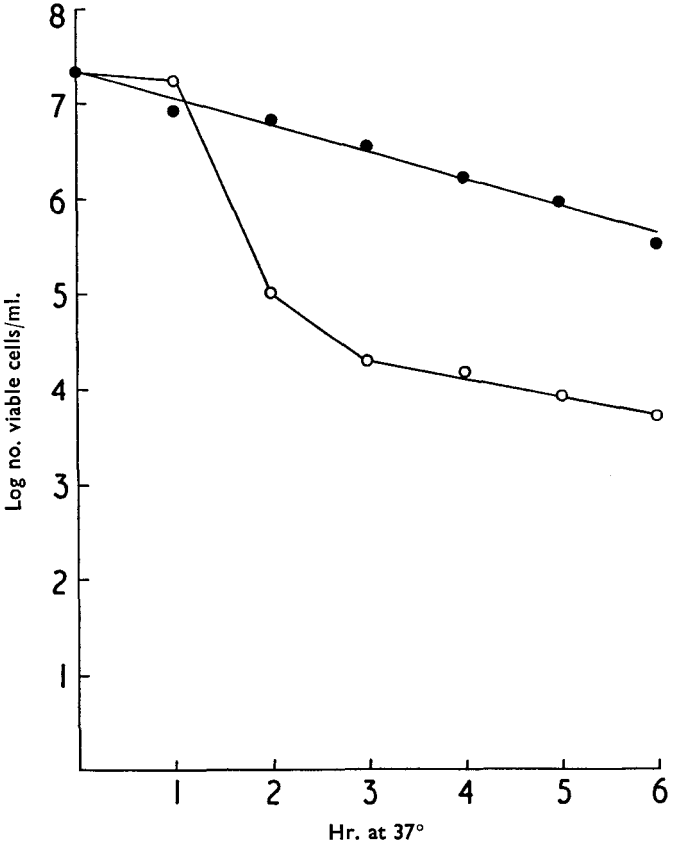

FIG. 2. Effect of incubation at 37° on the ability of spheroplasts of *E. coli* induced by 100 u/ml. penicillin to revert to the rod form.
●——● Colonies formed by spheroplasts.
○——○ Colonies formed by rods.

by Kellenberger and Ryter, 1958). These were: (i) Rigid (R) layer: innermost, rigid. (ii) Lipopolysaccharide layer: intermediate, soft. (iii) Lipoprotein layer: outermost, soft.

The R-layer was found to be composed of glutamic acid, alanine, DAP, glucosamine and muramic acid.

Thus, the accumulation of cell wall precursors (Park nucleotides), the decreased content of some constituents in the rigid layer of the cell wall and the morphological changes induced in bacteria can all be explained by the loss of integrity of the cell wall after the interruption of wall synthesis.

An interesting additional fact is that by growing certain bacterial species unable to synthesise DAP in media containing limiting amounts of this substance, morphological changes similar to those found with penicillin were observed (Meadow, Hoare and Work, 1957; McQuillen, 1958a, b).

Lederberg (1956) and Lederberg and St. Clair (1958) described the reversion of the penicillin-induced spheroplasts of *E. coli* to the rod form

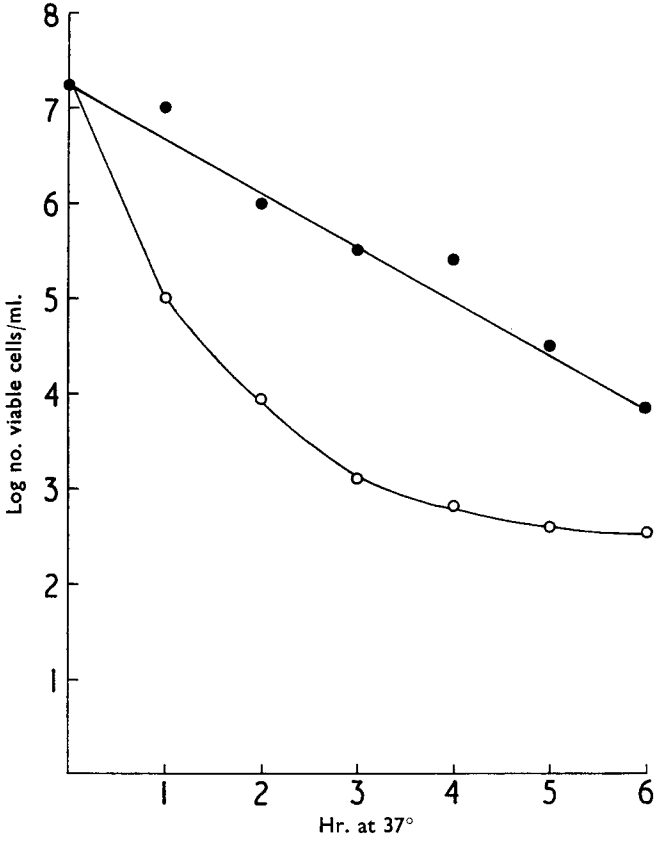

FIG. 3. Effect of incubation at 37° on the ability of spheroplasts of *E. coli* induced by 5000 u/ml. to revert to the rod form.

●——● Colonies formed by spheroplasts.
○——○ Colonies formed by rods.

on dilution into protective medium lacking penicillin, and Landman, Altenbern and Ginoza (1958) found that each intact spheroplast was capable of giving rise to either an L-form or a rod form. These observations on reversion to rods suggested that the spheroplasts were able to resynthesise a new rigid wall after removal of penicillin, an observation which had not been seen with lysozyme-induced protoplasts of Gram-positive bacteria.

Russell (1961) investigated the reconversion to rods of spheroplasts of *E. coli*, induced by 100 or 5,000 units/ml. penicillin, by diluting the

spheroplasts into penicillin-free protective media (A) or water (B) and plating into sucrose-Mg^{++} agar. Subtraction of colonies of B from those of A then gave the number of spheroplasts which were capable of engendering typical bacillary colonies. The results of such a determination (Figs. 2, 3) indicated that reversion of spheroplasts to rods was dependent on two factors; (i) penicillin concentration, and (ii) length of incubation.

Lederberg (1956, 1957) and Lederberg and St. Clair (1958) showed that there was a one-for-one conversion of rods into spheroplasts. Landman and others (1958), however, made the interesting point that only about 50 per cent of the original rods could survive as spheroplasts. By using synchronously-dividing cultures of *Alcaligenes faecalis*, Lark (1958b) showed that penicillin-induced crescent formation was dependent on the stage of growth in the synchronous cycle.

Hugo and Russell (1960a; 1961) investigated the effects of penicillin in hypertonic medium on *E. coli* and on an active penicillinase producer, *Cloaca cloacae*, and concluded that penicillin in high concentrations killed the cells by a mechanism other than that involving cell wall synthesis (as measured by spheroplast induction). In this connection, it is of interest to note that Smith, Payne and Watson (1960) were unable to induce spheroplasts by penicillin treatment in *Aerobacter* (*Cloaca*) *cloacae*. Spheroplasts were, however, induced by a modification of the lysozyme method of Zinder and Arndt (1956).

Hurwitz and others (1958) suggested that penicillin did not act solely by making the cells susceptible to its lytic action, and Prestidge and Pardee (1957) stated that the formation of spheroplasts could be objected to on the grounds that it was highly dependent on the external environment, and that the mechanism leading to sphere formation in hypertonic medium was not necessarily the same as that leading to death of the organism in a hypotonic medium. They further suggested that penicillin caused the formation of an enzyme which attacked the cell membrane and allowed the cell contents to escape.

Hugo and Russell (1960a; 1961) found that some bacteria were able to survive the action of high concentrations of penicillin, and showed that surviving cells of *E. coli* could not be classified as mutants. The presence of survivors was also shown by Landman and others (1958), but Hurwitz and others (1958) and Nozzolillo and Hochster (1959) reported the complete absence of persisting viable organisms, although the method adopted by the latter in this respect is open to criticism.

Spheroplast formation induced by penicillin has also been observed in Gram-positive bacteria, for example, in staphylococci (Murray, Francombe and Mayall, 1959) and in *Bacillus cereus* and *B. anthracis* (Foldes and Meretey, 1960).

It is of interest to note that penicillin-induced spheroplasts retain at least some of the biochemical capabilities of the rods from which they were derived (Sheinen and McQuillen, 1959; Russell, 1961).

Sedlaczek, Czerniawski and Zablocki (1958) found that the lipopolysaccharide-protein complex of *E. coli* had a specific protective effect on Gram-positive organisms against penicillin.

This leads to the concept that the two outer layers of the cell wall of *E. coli* prevent the antibiotic from reaching its site of action. In this context it is of interest to note the work of English, McBride and Huang (1960) who found that the resistance to penicillin of some bacteria was due to (*a*) penicillinase production or (*b*) penicillin acylase activity. A third group, however, were resistant to similar penicillin concentrations but possessed neither enzyme.

Baddiley and his colleagues (Baddiley, Buchanan, Carss, Mathias and Sanderson, 1956) isolated two nucleotides from *Lactobacillus arabinosus*. These were identified as cytidine diphosphate glycerol (Baddiley, Buchanan,

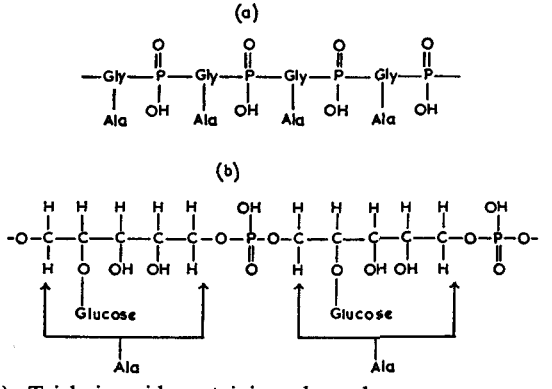

FIG. 4. (a) Teichoic acid containing glycerol
(b) Teichoic acid containing ribitol (the precise point of linkage of the alanine molecules is as yet undetermined).
Gly represents glycerol
Ala represents alanine.

Mathias and Sanderson, 1956) and cytidine diphosphate ribitol (Baddiley, Buchanan, Carss and Mathias, 1956; Baddiley, Buchanan and Carss, 1957). A compound containing ribitol phosphate was isolated from the cell wall of this organism (Baddiley, Buchanan and Greenberg, 1957) and ribitol phosphate was also shown to be present in large amounts in the cell walls of *B. subtilis* and *Staph. aureus* H, but not in *E. coli* and *Micrococcus lysodeikticus* (Baddiley, Buchanan and Carss, 1958).

Armstrong and others (1958) suggested the term "teichoic acids" (from the Greek "teichos" meaning "a wall") for these ribitol phosphate polymers.

It was found that cytidine diphosphate ribitol (but not cytidine diphosphate glycerol) accumulated in *Staph. aureus* treated with penicillin or crystal violet, which may again be thought of as an effect of penicillin in interfering with wall synthesis, and (this time) with teichoic acid production. Armstrong and others (1958) suggested that cytidine diphosphate glycerol was concerned in the synthesis of the protoplast membrane, since this has been shown (Mitchell and Moyle, 1956) to contain glycerophosphate.

In a recent investigation, however, Baddiley and Davison (1961) have shown that the cell walls of certain strains of lactobacilli contained a

glycerol teichoic acid, whilst a ribitol teichoic acid was found in the walls of other bacteria of this species.

Structure a (Fig. 4) is a compound containing glycerol and is the repeating unit of a teichoic acid from the walls of *B. subtilis* (Baddiley, 1960).

No mention has been made of the induction of L-forms by penicillin and other agents. The interested reader is referred to the recent review by Klieneberger-Nobel (1959).

Although the present review deals with the mode of action of penicillin, it is not out of place to mention the recent isolation of the penicillin "nucleus", 6-aminopenicillanic acid (6-APA) (Batchelor, Doyle, Nayler and Rolinson, 1959) from which it is hoped to prepare a wide range of semi-synthetic penicillins. It is of interest to note that both 6-APA and penicillins other than benzylpenicillin can also induce spheroplast formation in Gram-negative bacteria (Hugo and Russell, 1960b, c).

The many reported cytological changes of cell swelling, elongation, lysis, bacteriostasis and death, and the biochemical findings of the many workers in this complex field are, after twenty years of study, beginning to form a coherent pattern. The suppression of lysis by allowing penicillin to act on dividing cells in hypertonic medium was a vivid cytological demonstration of the action of the drug. Cell wall analyses and the detection of cell wall precursors in media in which penicillin was acting were of equal importance in the elucidation of the antibacterial mechanism.

As yet, the secondary action of penicillin has to be clearly elucidated, although possible mechanisms have been postulated.

In the field of structure-action relationships, it is of great interest that the parent amine, 6-aminopenicillanic acid, is itself capable of inducing effects on cell wall formation similar to those induced by benzylpenicillin.

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