The Interrelationship between Mucopeptide and Ribitol Teichoic Acid Formation as Shown by the Effect of Inhibitors

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1. The biosynthesis of teichoic acid in cell suspensions of two strains of *Staphylococcus aureus* is partially inhibited by the same low concentrations of penicillin that inhibit mucopeptide synthesis by 90–100%. Further increase in the concentration of the antibiotic by several hundred-fold still fails to cause any greater inhibition of teichoic acid synthesis. 2. Other conditions, such as amino acid deficiency or the presence of cycloserine or 5-fluorouracil, that inhibit mucopeptide synthesis also inhibit teichoic acid formation. 3. The degree of inhibition of teichoic acid synthesis caused by relatively high concentrations (10 µg./ml.) of benzylpenicillin depends critically on the age of the culture from which the cell suspensions have been prepared. 4. No significant amounts of soluble teichoic acid have been found in the fluid from cells incubated in the presence of penicillin. 5. A high proportion of the teichoic acid formed in the presence of penicillin can be removed from wall preparations at room temperature by 0.1N-ammonia. This is not true of the teichoic acid formed in the absence of penicillin. 6. The teichoic acid extracted with ammonia from preparations of cell walls made from cells treated with penicillin is excluded from Sephadex G-25, has a low molar ratio of glucosamine to phosphorus and contains muramic acid, alanine, glutamic acid, glycine and lysine. 7. The implications of these results for the mechanism of action of penicillin are discussed.

When whole living bacteria are studied under conditions such that growth cannot occur, the biosynthesis of mucopeptide in the cell walls of *Staphylococcus aureus* and *Escherichia coli* is inhibited by the penicillins at concentrations similar to those required to kill sensitive micro-organisms (Park, 1958; Mandelstam & Rogers, 1959; Shockman, 1959; Rogers, 1962; Rogers & Jelszewicz, 1961; Rogers & Mandelstam, 1962; Wylie & Johnson, 1962). The mucopeptides provide the necessary mechanical support to the delicate protoplasmic membrane of bacterial cells and give to micro-organisms their characteristic morphology, but they do not account for the whole material of the cell walls. For example, in staphylococci the mucopeptides account for about 50–60% of the dry weight of wall preparations, and in Gram-negative forms as little as 3–5% exists as this component (Mandelstam, 1962; Weidel & Pelzer, 1964). In staphylococci a high proportion of the rest of the wall consists of teichoic acid, a ribitol phosphate polymer with *N*-acetylglucosamine and *D*-alanine residues attached to the ribitol (Baddiley, Buchanan, RajBhandary & Sanderson, 1962a; Baddiley, Buchanan, Martin & RajBhandary, 1962b; Sanderson, Strominger & Nathenson, 1962).

The alanine is joined through a labile ester bond to either the 2- or 3-position of *D*-ribitol, leaving the 4-position of the polyol to which the *N*-acetylglucosamine is joined as an α - or β -glycoside. It seems probable that separate polymers with α - or β -linkages are involved (Torii, Kabat & Bezer, 1964), and the proportion of polymers with α - or β -anomers may alter according to the strain of organism (Baddiley *et al.* 1962a). The length of the polyribitol phosphate chain appears to vary from about nine to 14 units in the limited number of strains so far examined, although Strominger & Ghuyssen (1963) reported the presence of material of 40 units in fragments isolated from enzymic digests of the cell wall of *S. aureus* strain Copenhagen.

When growing staphylococci are treated with penicillin, CDP-ribitol accumulates (Saukkonen, 1961; Saukkonen & Virkola, 1963) as well as UDP compounds that are closely related in composition to the wall mucopeptide (Park, 1952; Park & Strominger, 1957; Strominger, 1962). The CDP-ribitol serves as a precursor of ribitol teichoic acid in cell-free systems prepared from *Lactobacillus arabinosus* (Glaser, 1963). Penicillin in rather high concentration has been shown to

cause a partial inhibition of the incorporation of inorganic [^{32}P]phosphate into the walls of staphylococci and *Bacillus subtilis* (Nathenson & Strominger, 1961; Roberts & Johnson, 1962) during growth of the organisms, yet, paradoxically, the antibiotic does not inhibit the cell-free systems synthesizing either polyribitol or mucopeptide (Glaser, 1964; Chatterjee & Park, 1964; Meadow, Anderson & Strominger, 1964). Nor does the antibiotic inhibit the addition of *N*-acetylglucosamine to α -*N*-acetylglucosaminylpolyribitol phosphate by a cell-free system from *S. aureus* (Nathenson & Strominger, 1963).

A possible explanation of this paradox is that in the staphylococcus the processes for the formation of teichoic acid, mucopeptide and of the linkage between these two polymers in the organized wall are interdependent. If this were true, then specific inhibition of the synthesis of either polymer would prevent the formation of the other. Alternatively, penicillin might specifically inhibit the process of linkage between preformed mucopeptide and teichoic acid already possibly linked to a small soluble mucopeptide, rather than the formation of either component. If this interpolymeric bond involves muramic acid, then there might be an explanation of the sensitivity to penicillin of species of organisms with various polymers other than teichoic acid joined to the mucopeptide in their cell walls.

The present paper demonstrates that inhibition of mucopeptide formation, due to a variety of causes, always leads to partial inhibition of teichoic acid synthesis. The nature of the small amount of teichoic acid that is still formed under these circumstances is also examined.

MATERIALS

Micro-organisms. *S. aureus* strains 524SC, Oxford and P20 were used. The strain P20 was obtained from Dr Mary Barber; the other two strains were as previously described (Mandelstam & Rogers, 1959; Rogers & Jeljaszewicz, 1961). Both strain Oxford and P20 were sensitive to 0.02–0.03 μg . of benzylpenicillin/ml.

Chemicals. Benzylpenicillin was Crystapen of Glaxo Laboratories, Greenford, Middlesex. Cycloserine (oxamycin) was from Eli Lilly and Co. Ltd., Basingstoke, Hants. 5-Fluorouracil was a kind gift from Hoffman-La Roche, mediated by Dr Morrison.

Radioactive materials. [^{32}P]Phosphate was carrier-free inorganic phosphate dissolved in dilute HCl from The Radiochemical Centre, Amersham, Bucks. The solution was diluted on receipt with sodium–potassium phosphate buffer, pH 7.0 (10 mM with respect to phosphate), so as to contain 100 μC /ml. [^{14}C]Alanine, DL-[^{14}C]glutamic acid, L-[^{14}C]lysine and [^{14}C]glucose were all from this same source. The amino acids were diluted with the appropriate unlabelled amino acid so that 1 mg. contained 1 μC of radioactivity, and were stored as a stock solution containing

1 mg./ml. The labelled glucose was dissolved in water soon after receipt and kept frozen at -20° .

METHODS

Growth of organisms

A dried culture was inoculated into 10 ml. of Hedley Wright broth contained in a 50 ml. flask, warmed to 35° , and incubated with shaking overnight. The next day an appropriate volume of the same medium was inoculated with this culture in the ratio 1:300. Unless otherwise stated this culture was grown until the extinction, measured with a Spekker absorptiometer, was equivalent to 1.0 mg. dry wt./ml. This culture was then kept at $0-4^\circ$ overnight. The next day it was warmed to 35° , diluted with an equal volume of warmed broth and grown with shaking until the extinction value was again equivalent to 1.0 mg./ml.

Preparation of suspensions

The culture was cooled, and the organisms were harvested by centrifuging at room temperature and washed once with water. The organisms were then suspended in a tris–maleate buffer, pH 7.0 (0.1 M with respect to maleate), containing 1% of glucose. The suspensions were finally adjusted so that they contained 2.0 mg. dry wt. of micro-organisms/ml.

Preparation of cell walls

The bacteria were broken by shaking for 20 min. in a Mickle disintegrator with ballotini beads, as described by Salton & Horne (1951). The unbroken cells were removed by centrifuging for 10 min. at 3000g. The cell walls were then deposited by centrifuging for 10 min. at 10000g. When the incorporation of [^{32}P]phosphate was studied, the walls were washed three times with sodium–potassium phosphate buffer, pH 7.0 (0.3 M with respect to phosphate), then three times with M-NaCl and twice with water. When ^{14}C -labelled amino acids or glucose had been used, the washings with phosphate were substituted by washings with solutions of the appropriate unlabelled compound (1 mg./ml.). All the centrifuging was done at $0-4^\circ$. This is important, to avoid losses of cell-wall material due to activity of the lytic enzyme present in the preparations. Finally, the preparations were dried from the frozen state.

Biosynthesis of teichoic acid

Micro-organisms equivalent to 1 mg. dry wt./ml. were suspended in a solution containing the following (final concentrations given): DL-alanine, DL-glutamic acid, L-lysine and glycine (each 0.2 mg./ml.), sodium–potassium phosphate buffer, pH 7.0 (15 mM with respect to phosphate), glucose (27 mM) and tris–maleate buffer (0.1 M with respect to maleate and 0.05 M with respect to tris; adjusted to pH 7.0 with NaOH). Then 1 μC of [^{32}P]phosphate/ml., 0.02 μC of ^{14}C -labelled amino acid/ml. or 0.15 μC of [^{14}C]glucose/ml. was added, whichever was appropriate. When labelled glucose was used the concentrations of unlabelled hexose were reduced to 2.7 mM final concentra-

tion. Such a suspension (100 ml.) was then incubated in a 2 l. conical flask, lightly plugged with cotton wool and shaken at the maximum throw of a shaker of the type designed by Kantorowicz (1951). After the required time of incubation the flasks were removed and cooled rapidly in ice-cold water, and the suspensions centrifuged and washed once with 0.1M-sodium-potassium phosphate buffer, pH 7.0. Finally, the organisms were suspended at about 20 mg./ml. in water for the preparation of cell walls.

Subsequent procedure with [^{32}P]phosphate or [G - ^{14}C]glucose. The cell-wall preparations (5–10 mg.) were suspended in 1.0 ml. of 5% (w/v) trichloroacetic acid and placed in a water bath at 90° for 15 min. The residue was removed by centrifuging at 5000g, resuspended in 1.0 ml. of 5% trichloroacetic acid and centrifuged again. The original supernatant and the washing fluids were combined. The trichloroacetic acid was removed by three extractions each with 5.0 ml. of diethyl ether. The ether was removed by passing a stream of air through the solution warmed to 40°. Finally the volume was adjusted to 5.0 ml. One sample from this solution was dried on a planchet for radioactive counting, and another for the determination of total phosphorus, when [^{32}P]phosphate had been used; the specific activity of the phosphorus could then be calculated.

Alternative subsequent procedure with [^{14}C]alanine. The cell-wall preparations (about 4 mg.) were suspended either in water (400 μl .) with 0.01N-NaOH added to adjust the pH to 10–11, or in 1.0N-NH₃ (500 μl .) at room temperature for 5 hr. When NaOH had been used, the suspensions were centrifuged and samples of the supernatant were applied to Whatman no. 3 paper. Alanine was separated and its specific activity determined as described under 'Measurement of mucopeptide synthesis' below. When aq. NH₃ had been used the suspensions were centrifuged and the residues washed twice with water. The supernatants and washings were combined and dried *in vacuo*. The dried material was taken up in 2N-HCl (1.0 ml.) and heated at 105° for 2½ hr. to decompose alanine amide. The hydrolysate was evaporated to dryness, redissolved in water twice, and each time evaporated to dryness over NaOH and anhydrous CaCl₂. The specific activity of the alanine was then determined after separation by paper chromatography.

Isolation of hexosamines. The ^{14}C -labelled cell-wall fractions were hydrolysed in 4N-HCl at 105° for 4 hr., dried *in vacuo* over NaOH and then redissolved in water. Glucosamine and muramic acid were separated on charcoal-Celite columns (Rogers & Perkins, 1959), and glucosamine was separated from free amino acids by paper chromatography of the concentrated aqueous eluate, as described in the section on the determination of the specific activity of amino acids under 'Measurement of mucopeptide synthesis'. A portion of this fraction was counted at infinite thinness, and the glucosamine content was determined by the method of Rundle & Morgan (1955). In a similar way muramic acid was isolated from the 10% (v/v) ethanolic eluate from the charcoal-Celite fractionation of the mucopeptide hydrolysate.

Isolation of teichoic acid. This was done from wall preparations by using cold 10% (w/v) trichloroacetic acid, as described by Baddiley *et al.* (1962a). In some instances the whole extract made with cold trichloroacetic acid was used, whereas in others the ethanolic precipitation procedure of Baddiley *et al.* (1962a) was also used. When the whole extract was used the trichloroacetic acid was removed by

extraction with diethyl ether before the extract was examined further.

Measurement of mucopeptide synthesis

This was measured by the use of either DL-[^{14}C]alanine, DL-[^{14}C]glutamic acid or L-[^{14}C]lysine. The suspension fluid for incubation of the organisms contained DL-alanine, DL-glutamic acid, L-lysine, glycine, glucose, phosphate and tris-maleate buffer, as described under 'Biosynthesis of teichoic acid'. The conditions for incubation are also as described there, and, apart from the substitution of the tris-maleate buffer for a phosphate buffer, are closely similar to those used in previous work (Mandelstam & Rogers, 1959). Chloramphenicol was not included. When DL-[^{14}C]alanine had been used, the ester alanine attached to teichoic acid was first removed from the cell-wall preparations either by treating them with 1.0N-NH₃ for 5 hr. at room temperature or by heating them with 5% trichloroacetic acid for 15 min. at 90° (the latter procedure also removes the polyribitol phosphate of the teichoic acid). In either case the insoluble material that remained was then washed and dried with ethanol and ether. When [^{14}C]glutamic acid or [^{14}C]lysine had been used, the pretreatment with aq. NH₃ or hot trichloroacetic acid was not necessary. A suitable sample (1–2 mg.) of the cell walls was then mixed with 1.0 ml. of 6N-HCl and heated overnight at 105° in a sealed ampoule. The HCl was removed *in vacuo* over NaOH pellets and anhydrous CaCl₂, and the residue was twice taken up in water and evaporated to dryness. Finally, the hydrolysate was applied as a streak to Whatman no. 3 paper and the amino acids were separated by chromatography, with butanol-acetic acid-water (63:10:27, by vol.; upper phase), along with marker amino acids. After drying the paper, the relevant amino acid was located by applying a thin line of 0.1% ninhydrin down the paper at the edge of the applied streak. The necessary area of paper was cut out and eluted with water. Part of the eluate was used for amino acid estimation, and the remainder was dried on a metal planchet and counted. From these measurements the specific activity of the amino acid was determined.

Sephadex G-25 fractionation

The fine material from Sephadex G-25 was thoroughly removed by decantation and the larger material suspended in water and run into a column (68 cm. $\times$ 3.5 cm.) by the method described by Flodin (1962). The column was then thoroughly washed with 0.1N-NH₃ until titration of the inflowing and outflowing solutions required equal volumes of 0.1N-HCl for neutralization. The samples were then applied in solution in 2.0 ml. of 0.1N-NH₃ and washed into the column with two further 2.0 ml. lots of 0.1N-NH₃. The flow of 0.1N-NH₃ was continued, 6.0 ml. fractions were collected, and the radioactivity in 0.2 ml. of the samples was measured at infinite thinness after the material had been dried on metal planchets. The exclusion volume (Flodin, 1962) of the column was measured by applying a solution of 20 mg. of bovine plasma albumin (fraction V from Armour Laboratories, Eastbourne, Sussex) dissolved in 2.0 ml. of 0.1N-NH₃. Fractions (6.0 ml.) were collected and their extinction values at 280 m μ used to determine the albumin concentration.

Analytical procedures

Radioactive determinations. For the measurement of all samples except those obtained during Sephadex fractionation, a thin end-window Geiger-Müller tube and scaler was used with an efficiency such that $1\mu\text{C}$ of ^{14}C gave 2×10^5 counts/min. and $1\mu\text{C}$ of ^{32}P gave 10^6 counts/min. The error of counting was $\pm 5\%$, 1000 counts above background being collected. For the fractions from Sephadex columns a Nuclear-Chicago gas-flow instrument was used with a windowless tube.

Chemical analyses. Phosphorus was measured as by Chen, Toribara & Warner (1956). Amino acids were separated and measured as by Mandelstam & Rogers (1959). Glucosamine was measured, after hydrolysis for 4 hr. with 4N-HCl at 105° in sealed ampoules and removal of the HCl *in vacuo* over NaOH pellets and anhydrous CaCl_2 , as by Rondle & Morgan (1955).

RESULTS

Biosynthesis of teichoic acid in the absence of antibiotics

The most unambiguous way of measuring the synthesis of teichoic acid by bacterial cells, provided that no turnover takes place, would be to estimate increase in the amount of teichoic acid phosphate in the walls. Since mucopeptide formation also occurs, increases in the proportion of teichoic acid in the wall preparations would not be expected. Therefore it would be necessary to measure the total teichoic acid in the walls of the organisms, which would involve obtaining a quantitative recovery of wall material. This, however, is not practicable, first because of the uncertainties of quantitative yields of wall during the removal of whole cells by differential centrifugation, and secondly because of loss of wall material due to degradation by the lytic enzymes of the cell that are active during the preparative manipulations. In previous work (Mandelstam & Rogers, 1959) this latter complication was avoided by heating the wall preparations at an early stage of their preparation. In the present work this was thought inadvisable because it might in some instances lead to loss of teichoic acid, but the action of lytic enzymes was minimized by preparing the walls at $0-4^\circ$. In view of such difficulties with a direct method of measuring teichoic acid synthesis, the more indirect method was used of measuring the incorporation of either $[^{32}\text{P}]$ phosphate into the poly-ribitol 'backbone', $[1-^{14}\text{C}]$ alanine into ester linkage or $[U-^{14}\text{C}]$ glucose into *N*-acetylglucosamine and ribitol. The ester-linked alanine could be readily separated quantitatively from the alanine in the mucopeptide by treatment of the walls at room temperature or 0° with dilute alkali (Archibald, Armstrong, Baddiley & Hay, 1961; Shockman, 1963). Such a separation could thus allow measure-

ment of the synthesis of both teichoic acid and mucopeptide from the incorporation of one amino acid.

In preliminary experiments it was shown that the synthesis of mucopeptide by *S. aureus* strain Oxford was not influenced by differences in phosphate concentration between 0.1mM and 0.1M added to the tris-maleate buffer (see the Methods section under 'Biosynthesis of teichoic acid'). The incorporation of $[1-^{14}\text{C}]$ glutamic acid into mucopeptide during 90 min. was the same when the tris-maleate buffer containing phosphate (0.1mM) was used as it was with the phosphate-glucose buffer of previous work (Mandelstam & Rogers, 1959). When the course of incorporation of $[^{32}\text{P}]$ -phosphate was studied with tris-maleate buffer with or without added carrier inorganic phosphate (30mM), the specific activity of the total phosphate extracted from the walls by hot trichloroacetic acid rose to a peak at about 30 min. and then declined. In one experiment this phenomenon

Fig. 1. Time-course for the incorporation of ^{32}P into cell-wall material by suspensions of *S. aureus*. The suspensions were made in a solution of the composition described in the Methods section. Separate 21. flasks each containing 100 ml. of suspension were shaken at 35° and a flask was removed at each of the time-intervals indicated. Cell walls were prepared and extracted with 5% trichloroacetic acid, and the specific activity of the ^{32}P was estimated as described in the Methods section.

appeared to be slightly more pronounced in the presence of chloramphenicol than in its absence, and in view of this observation chloramphenicol was omitted from the solution used for incubation in the rest of the work described below. These preliminary experiments had been conducted in conical flasks of a volume five times that of the suspensions. By chance it was found that when less suspension was added to larger flasks (cf. legend to Table 1), thus greatly increasing the degree of aeration, incorporation of [32 P]phosphate became approximately linear with time (Fig. 1). The reasons for the peak of incorporation under conditions of poorer aeration have not been investigated further. In all further work 100 ml. volumes of suspension were shaken in 2 l. conical flasks.

Relationship of the phosphorus soluble in hot trichloroacetic acid to teichoic acid

The possibility of rapid incorporation of [32 P]-phosphate into a small proportion of the total phosphorus present in the walls as some non-teichoic acid compound that is also dissolved by hot trichloroacetic acid had first to be excluded. The specific activity of the phosphorus in hot trichloroacetic acid extracts was therefore compared with that in teichoic acid samples extracted from the walls with cold 10% trichloroacetic acid and precipitated by ethanol. These samples were further purified on a column of DEAE-cellulose and the specific activities of the materials in the peak were again measured. For this experiment cells were incubated for 30 and 60 min. in the presence of [32 P]phosphate in the suspension fluid described in the Methods section. For the column chromatography, 0.5 ml. samples containing 55 and 65 μ g. of phosphorus were applied to columns (5 cm. $\times$ 1 cm.) of DEAE-cellulose equilibrated with 0.01 M-ammonium acetate buffer, pH 6.5. The concentration of the ammonium acetate buffer flowing through the column was then increased in a stepwise fashion from 0.01 M through 0.05 M to 0.2 M. The highest concentration removed the radioactivity in each sample as a single peak; the samples corresponding to the top of the peak were pooled and dried *in vacuo*.

As shown in Table 1, the most highly active samples were those obtained by DEAE-cellulose fractionation of the isolated teichoic acid. This seems to indicate that the cold-trichloroacetic acid-extracted material has in it some rather poorly labelled molecules that are excluded from the main peak during chromatography. It may be pointed out that the relatively short period used for extraction of the walls (i.e. a total of 72 hr.) removes only part of the teichoic acid, and that the total yield of phosphorus was only about 20% by this process. Thus it is potentially a rather selective

Table 1. *Comparison of the specific activities of the phosphorus in hot- and cold-trichloroacetic acid extracts from cell walls with that in teichoic acid purified by chromatography*

S. aureus was grown and cell suspensions were prepared as described in the Methods section. Each incubation system consisted of 400 ml. of suspension (2 mg./ml.), 8 ml. of solutions (each 10 mg./ml.) of DL-alanine, DL-glutamic acid, L-lysine and glycine, phosphate (0.02 M; 180 μ C of 32 P), glucose (27 mM) and tris-maleate buffer, pH 7.0, adjusted to a total final volume of 800 ml., contained in a 5 l. conical flask. Incubation was at 35° with shaking. At 30 and 60 min. flasks were removed, the contents centrifuged and the cells washed once. Cell walls were prepared and yielded 49.6 and 61.0 mg. respectively after 30 and 60 min. incubation. Samples (3.6 and 4.4 mg.) were extracted with 5.0 ml. of 5% trichloroacetic acid for 15 min. at 90°. The remainder was suspended in cold 10% trichloroacetic acid and the teichoic acid isolated from the extract by ethanol precipitation. Samples were taken for analysis and the remainder was subjected to chromatography on DEAE-cellulose.

Sample	Time of incubation (min.) ...	10 ⁻⁴ $\times$ Sp. activity of 32 P (counts/min./mg. of P)		Ratio B/A
		30 (A)	60 (B)	
Hot-trichloroacetic acid extract		5.9	10.4	1.77
Cold-trichloroacetic acid extract		4.3	8.2	1.9
Teichoic acid after chromatography		7.1	15.9	2.2

process. The process of extraction with hot trichloroacetic acid does not yield material that is much more highly labelled than purified teichoic acid, but rather removes some phosphorus-containing substances that are rather poorly labelled, since the specific activity of the phosphorus in the hot-trichloroacetic acid extract was smaller than that obtained from chromatography. The degree of increase in specific activity between 30 and 60 min. incubation is similar for the material extracted by either hot or cold trichloroacetic acid and for the purified teichoic acid samples. Nevertheless, the experiment might be interpreted to show that the labelled teichoic acid deposited in the wall under the conditions chosen for incubation with [32 P]phosphate does not behave in exactly the same way under all circumstances as that already present in the walls of the organisms, and therefore not labelled. The unlabelled teichoic acid was, of course, formed during exponential growth of the bacteria in ordinary media. The importance of this point becomes more obvious below.

Tests were also made for the presence of nucleotides in the material extracted with hot trichloroacetic acid. The ultraviolet-absorption spectrum

showed no band between 250 and 280 m μ . Calculation from the extinction value at 260 m μ showed that less than 5% of the phosphorus would be due to nucleotide.

Formation of teichoic acid in the presence of inhibitors

Effect of penicillin and cycloserine. Suspensions (100 ml.) of *S. aureus* strains Oxford and P20 were incubated in flasks (21.) with [32 P]phosphate in the system already described above. Benzylpenicillin (2 μ g./ml.) or cycloserine (67 μ g./ml.) was present. The flasks were incubated for 30 and 60 min. At the end of these times cell walls were isolated, hot-trichloroacetic acid extracts made and the specific activities of the phosphorus in them determined. The incorporation of [32 P]phosphate, both in the presence and absence of inhibitors, proceeded in a linear fashion. Table 2 shows that penicillin at this concentration inhibited the incorporation of [32 P]phosphate by 40–50%, according to the time of measurement, and cycloserine by 65%, irrespective of the time of incubation. In another experiment the effect of 50, 100 and 200 μ g. of cycloserine/ml. was examined during 60 min. incubation, and a higher inhibition (78–81%) was obtained with 100 and 200 μ g./ml. Mucopeptide formation in this experiment was inhibited by 78–86% by these concentrations of cycloserine.

The effects of different concentrations of penicillin on teichoic acid and mucopeptide formation were then examined in greater detail. Teichoic acid formation was measured in separate experiments by the incorporation of either [32 P]phosphate or [14 C]alanine. Fig. 2 shows the results obtained with both the Oxford and P20 strains: synthesis of mucopeptide as measured by specific activity determinations on the alanine is inhibited by more than 90% by 0.2–0.3 μ g. of the antibiotic/ml. in both strains. At this concentration teichoic acid

formation estimated by [32 P]phosphate incorporation is inhibited by about 25–30% in strain Oxford and by 60–70% in strain P20. Results given below show that this probably only appears by chance as a strain difference, and is probably due to other small differences between the suspensions. Measured by incorporation of [14 C]alanine into ester-linked amino acid, teichoic acid synthesis in both strains is inhibited by about 40% by 0.1–0.3 μ g. of penicillin/ml. Increasing the concentration of the inhibitor by 100-fold up to 10 μ g./ml. has little further effect on teichoic acid synthesis. Thus very low concentrations of penicillin have some effect, but this does not increase proportionally with the concentration of the antibiotic. The apparent difference between the results for the incorporation of [32 P]phosphate and [14 C]alanine into the teichoic acid fraction is somewhat greater than would be expected on the basis of experimental error.

A similar result has been obtained with *S. aureus* strain 524SC, with methicillin. This organism produces penicillinase, and incomplete inhibition of mucopeptide synthesis by suspensions is obtained

Table 2. *Effect of benzylpenicillin and cycloserine on teichoic acid formation measured by the incorporation of [32 P]phosphate*

The effect of the two antibiotics was examined in separate experiments and the results are given for the two controls.

Time of incubation (min.)	$10^{-4} \times$ Sp. activity of ^{32}P (counts/min./mg. of P)			
	...	30	60	
No antibiotic (control)	5.8	3.4	8.6	5.32
Benzylpenicillin (2 μ g./ml.)	2.8	—	5.0	—
Cycloserine (67 μ g./ml.)	—	1.2	—	1.9

Fig. 2. Inhibition of the formation of teichoic acid and mucopeptide by increasing concentration of benzylpenicillin. The preparation of the suspensions and the measurement of incorporation of ^{32}P into the teichoic acid were as described in the Methods section. Both teichoic acid and mucopeptide synthesis were measured by the incorporation of [^{14}C]alanine into the ester-linked alanine fraction for the former and into the material insoluble in both trichloroacetic acid and alkali for the latter. Continuous lines represent the results for *S. aureus* strain Oxford, broken lines those for *S. aureus* strain P20; ● and ○, ^{32}P incorporation; ▲ and △, incorporation of [^{14}C]alanine into ester alanine; ■ and □, incorporation of [^{14}C]alanine into mucopeptide.

even with very large doses of benzylpenicillin (Mandelstam & Rogers, 1959; Rogers, 1962), owing to the hydrolysis of the inhibitor by penicillinase associated with the cells. However, a concentration of 2 $\mu\text{g.}$ of methicillin/ml. is sufficient to stop mucopeptide synthesis (Rogers & Jeljaszewicz, 1961) by this organism, but, with 200 $\mu\text{g./ml.}$, although mucopeptide synthesis measured by the incorporation of L-[U- ^{14}C]lysine was 97% inhibited, the incorporation of [^{32}P]phosphate into the material extracted with hot trichloroacetic acid from the walls was only stopped by 76%.

The effect of 10 $\mu\text{g.}$ of benzylpenicillin/ml. on the incorporation of radioactivity from [U- ^{14}C]glucose was also examined. The result obtained was similar to that with [^{32}P]phosphate and DL-[1- ^{14}C]alanine. The incorporation of radioactivity into the fraction from the walls that is soluble in trichloroacetic acid at 90° was inhibited about 60–70%, whereas that into the insoluble residue of mucopeptide was inhibited by about 90%, a result confirmed by measuring the specific activity of muramic acid isolated after acid hydrolysis of the mucopeptide. In a separate experiment in which the cells were incubated for 30 min. the glucosamine was isolated from both the insoluble mucopeptide and the material in the walls rendered soluble by hot trichloroacetic acid, and specific activities were determined. That of the glucosamine in the mucopeptide was 17.8 counts/min./ μmole , whereas that in the solubilized material was 65.5 counts/min./ μmole . If equal amounts of *N*-acetylglucosamine had gone from a single pool of a common precursor to both mucopeptide and teichoic acid, then the specific activity of the glucosamine from these compounds should have been inversely related to the total amount of amino sugar present in both after incubation. Since most of the amino sugar in both fractions will be from unlabelled

material already present in the cells before incubation, the ratio should be close to the amount of glucosamine in teichoic acid/amount of glucosamine in mucopeptide in the original cell walls. This ratio is about 0.6, whereas the inverse ratio of the specific activities for the amino sugar in the two fractions is 0.27, a result consistent with teichoic acid synthesis having gone on to a greater extent than that of mucopeptide.

Effect of age of cells on inhibition of [^{32}P]phosphate incorporation by penicillin. In studying the effect of penicillin during a number of experiments at different times, a considerable variation was noticed in the extent of inhibition caused by the antibiotic. Examination was then made of the relationship between the age of the culture from which the cells have been harvested and the extent of inhibition. This experiment was done by incubating broth cultures (see the Methods section) long enough for the cell densities to have reached the values indicated in Table 3. The flasks were removed from the incubator, cooled rapidly and kept at 0–4°. The next day they were diluted with an equal volume of medium and incubated until the density had again reached the point at which they had been removed from the incubator. Suspensions were prepared and tested for teichoic acid synthesis in the presence and absence of 10 $\mu\text{g.}$ of penicillin/ml., with [^{32}P]phosphate incorporation into the material soluble in hot trichloroacetic acid being used as an indicator of synthesis. There is a very marked reduction in the degree of inhibition with increasing cell density in the parent culture (Table 3). Thus the differences between the two strains, P20 and Oxford, shown in Fig. 2 are probably not due to intrinsic strain differences but to small differences in the density to which the cultures were grown before the bacteria were harvested. Likewise the day-to-day differences in

Table 3. *Effect of age of culture on inhibition by penicillin*

The cultures were incubated until the cell densities had reached the points indicated in the first column, cooled, and the next day diluted with an equal volume of medium and reincubated until the cell densities were again as indicated in the first column. Suspensions were prepared and teichoic acid synthesis was measured as described in the Methods section, with [^{32}P]phosphate. The presence of 10 $\mu\text{g.}$ of benzylpenicillin/ml. during incorporation is indicated by +, its absence by –.

Cell density on harvesting (mg. dry wt./ml.)	Benzylpenicillin	10 ⁻³ × Sp. activity of whole cell wall (counts/min./mg. dry wt.)	10 ⁻⁵ × Sp. activity of ^{32}P (counts/min./mg. of P)	Inhibition (%)	
		(a)	(b)	From (a)	From (b)
0.76	–	2.4	1.1	—	—
	+	0.9	0.4	63	64
1.22	–	2.3	1.1		
	+	1.0	0.5	53	58
1.46	–	3.1	1.4		
	+	2.3	0.9	27	39

degrees of inhibition caused by penicillin were likely also to be explained in the same way. In most of the work that follows below, the cultures were grown until the dry weight of cells was 1.1–1.2 mg./ml.

Search for soluble teichoic acid formed during penicillin inhibition. A simple explanation of the partial inhibition by penicillin of the incorporation of [32 P]phosphate or [1- 14 C]alanine into newly formed teichoic acid bound to the cell wall would be that, in the absence of mucopeptide formation, teichoic acid synthesis goes on, but that the substance is secreted into the surrounding fluid instead of being retained by the cell. To test this possibility the supernatant fluid was obtained from 600 ml. of suspension of cells of strain Oxford that had been incubated for 30 min. in the usual system in the presence of 10 μ g. of benzylpenicillin/ml. and 1 μ C of [32 P]phosphate/ml. The clear fluid was concentrated *in vacuo* at 30° to 60 ml., dialysed for 3 hr. against 1 l. of water, with rocking, concentrated to dryness and dissolved in 5.0 ml. of 0.1N-acetic acid. The dialysis treatment was included to reduce the amount of [32 P]phosphate and was not prolonged in order to avoid loss of teichoic acid. The final material was applied to a column of Sephadex G-25 (68 cm. $\times$ 3.5 cm.), previously washed with 0.1N-acetic acid. The flow of 0.1N-acetic acid through the column was continued and 8.5 ml. fractions were collected. A sample (0.2 ml.) of each fraction was then counted. A small peak of radioactivity emerged at a volume of 230 ml. The exclusion volume of the column was 204 ml. The radioactivity in this small peak corresponded to 5% of that in the teichoic acid associated with the wall preparation made from the organisms. Total phosphorus in the peak corresponded to 7.0% of that in the wall preparation. The small peak was followed at an elution volume of 400–500 ml. by a large peak formed by the remaining 32 P activity. There was 150 ml. of eluent between these peaks, containing no detectable radioactivity. Hydrolysis of the material in the small peak showed the presence of mucopeptide constituents and anhydro-ribitol. The amount of 32 P activity associated with teichoic acid in the supernatant fluid is thus far too small to account for the degree of inhibition (about 50%) of incorporation caused by the penicillin. Therefore leakage of freshly formed teichoic acid does not seem to be a likely explanation of the partial inhibition of the incorporation of [32 P]phosphate into the wall material.

Inhibition by 5-fluorouracil. It has been shown (Rogers & Perkins, 1960; Brockman & Chambers, 1962) that 5-fluorouracil can inhibit the biosynthesis of mucopeptide in staphylococci and cause the accumulation of 5-fluoroUDP-muramyl-peptides. The effect of 1.5 μ moles of 5-fluorouracil/ml. on

Table 4. *Inhibition of mucopeptide and teichoic acid synthesis by 5-fluorouracil*

S. aureus strain Oxford was grown and the cell suspensions were prepared in the usual way. They were incubated in the tris-maleate buffer system described in the Methods section, supplemented with 5-fluorouracil and cytidine as shown below.

Addition	10 ⁻⁴ $\times$ Sp. activity of P (counts/min./mg. of P)	10 ⁻³ $\times$ Sp. activity of glutamic acid (counts/min./mg.)
None	5.1	5.1
5-Fluorouracil (1.5 μ moles/ml.)	2.8	1.6
5-Fluorouracil (1.5 μ moles/ml.) + cytidine (1.0 μ mole/ml.)	2.5	2.3

the biosynthesis of mucopeptide and teichoic acid was examined. The incorporation of [1- 14 C]-glutamic acid was used to measure biosynthesis of mucopeptide and [32 P]phosphate for teichoic acid biosynthesis. The specific activity of the isolated glutamic acid was reduced by 69% by the presence of 5-fluorouracil and the incorporation of [32 P]-phosphate was inhibited by 45% (Table 4). The attempt to reverse specifically the inhibition of teichoic acid formation by the addition of cytidine was unsuccessful. Mucopeptide synthesis was somewhat greater in the presence of the nucleoside.

Inhibition of teichoic acid formation by amino acid deficiency. It was previously shown (Mandelstam & Rogers, 1959) that omission of cell-wall amino acids from the suspension fluid used with *S. aureus* strain 524 SC led to diminished synthesis of mucopeptide. The effects with *S. aureus* strain Oxford were very much less marked, and consequently strain 524 SC was used for these experiments. The effect of leaving out alanine, glutamate and glycine on mucopeptide and teichoic acid biosynthesis is shown in Table 5: the incorporation of L-[U- 14 C]lysine into mucopeptide is reduced by 70–80%; at the same time the incorporation of [32 P]phosphate into teichoic acid is also reduced by about 60%. Likewise, when only DL-alanine was present, mucopeptide and teichoic acid were both inhibited by 52 and 55% respectively.

Nature of material formed in the presence of penicillin

Removal of newly synthesized material from walls of penicillin-treated cells. When wall preparations from staphylococci not incubated with penicillin are treated with alkali (either 0.1N-ammonia or 0.01N-sodium hydroxide, the ester-linked alanine of the teichoic acid they contain is liberated quan-

Table 5. *Effect of amino acid deficiency on teichoic acid formation*

A cell suspension of *S. aureus* strain 524SC was prepared in the usual way and incubated either in the presence of DL-alanine, DL-glutamic acid, L-lysine and glycine or with either L-lysine or DL-alanine alone. One set of flasks contained 0.01 μ C of L-[U- 14 C]lysine or [1- 14 C]alanine/ml. and the other 1 μ C of [32 P]phosphate/ml. Incubation was for 30 min. For estimation of mucopeptide synthesis the infinitely thick preparations of cell walls were counted. For teichoic acid synthesis the specific activity of phosphorus extracted from cell-wall preparations by 5% trichloroacetic acid for 15 min. at 90° was estimated. N.D., Not determined.

Expt. no.	Suspension medium	Mucopeptide synthesis (counts/min.)	Inhibition of mucopeptide synthesis (%)	Teichoic acid synthesis		Inhibition of teichoic acid synthesis (%)
				10 ⁻³ × Sp. activity of whole cell wall (counts/min./mg. dry wt.)	10 ⁻⁴ × Sp. activity of 32 P (counts/min./mg. of P)	
I	Complete	226	—	1.26	7.6	—
	L-Lysine only	64	76	0.49	2.9	62
II	Complete	515	—	N.D.	8.0	—
	DL-Alanine only	249	52	N.D.	3.6	55

Table 6. *Alkaline extraction of phosphorus from cell walls of S. aureus*

Cell walls were prepared from cells of *S. aureus* strains Oxford and P20 that had been incubated in tris-maleate buffer, pH 7.0, at 35°. Benzylpenicillin (final concn. 10 μ g./ml.) and [32 P]phosphate were included in the incubation mixtures as indicated below. Freeze-dried samples of cell walls were suspended in 0.1N-NH₃ or N-NH₃ for 5 hr. at 20°, and the radioactivity and total phosphorus extracted were measured. All values given for 32 P have been corrected for radioactive decay. Experimental details are given in the text.

Expt. no.	Strain	Additions		Time of incubation of cells (min.)	10 ⁻³ × 32 P incorporated into cell wall (counts/min./mg. dry wt.)	Percentage of 32 P extracted with NH ₃	Percentage of total P extracted with NH ₃
		[32 P]-Phosphate	Benzylpenicillin				
1	Oxford	+	—	30	2.03	15	—
2	Oxford	—	—	*	—	—	19
3	Oxford	+	+	15	1.58	80	8
				40	2.01	90	11
				60	3.41	92	19
4	Oxford	+	+	30	1.04	94	16
5	Oxford	+	+	60	1.36	89	14
6	P20	+	—	30	0.84	18	—
7	P20	+	+	30	0.33	66	20

* Walls were isolated from cells that had not been incubated in the system used for studying mucopeptide and teichoic acid synthesis.

titatively as either a mixture of free amino acid and its amide or as free alanine (Archibald *et al.* 1961; Shockman, 1963). Very little more of the phosphorus-containing 'backbone' of the polymer is removed by alkali than by water; in frozen-dried preparations of walls a little (10–20%) has always been found to be soluble. These observations have been fully confirmed here for the radioactively labelled teichoic acid (cf. Table 6). However, when wall preparations from cells that had been incubated in the presence of penicillin and [U- 14 C]glucose or

[32 P]phosphate were treated with 0.1N-ammonia for 5 hr. at 20°, not only the ester-linked alanine but a very high proportion of the incorporated label was also removed (Table 6). However, only a small proportion of the total phosphorus in the wall was removed under these conditions. It is important to note that, when [U- 14 C]glucose has been used, the total counts extracted from equal weights of cell wall from organisms incubated in the presence and absence of penicillin are similar, even though the proportion of the radioactivity extracted from the

penicillin-treated cells is much higher. Attempts were made to remove the labelled phosphate from the walls of cells incubated in the presence of penicillin by treating them for 2 hr. at 19° with 0.1–2.5 M-ammonium acetate and 8.0 M-urea. The highest concentration of the salt and the urea solution both removed 25% of the radioactivity, whereas similar treatment with water removed 16%.

Constituents. Acid hydrolysis (2N-hydrochloric acid at 100° for 3 hr.) of the total material isolated by treating wall preparations made from cells incubated in the presence of 10 µg. of penicillin/ml. with 1.0N-ammonia showed that anhydrosorbitol and glucosamine were present in the material rendered soluble. Estimation of the phosphorus:glucosamine molar ratios on four preparations gave values of 1:0.3, 1:0.5, 1:0.6 and 1:0.3. These values for the ratios were rather low and variable. When teichoic acid, isolated by extraction with cold 10% trichloroacetic acid from cell walls of organisms grown in the ordinary way, was examined, results of 1:0.7 and 1:0.6 were obtained.

Fractionation of ammonia extracts. Cell walls (70 mg.) from cells incubated in the presence of 10 µg. of benzylpenicillin/ml. and [U-¹⁴C]glucose were extracted with 0.1N-ammonia (10 ml.) for

5 hr. at 20°. The resulting extract was applied to a column of Sephadex G-25. The column was then eluted with 0.1N-ammonia and samples from the fractions were examined for radioactivity (Fig. 3). The large peak corresponded to the position occupied by serum albumin when this was run through the column, and can therefore be regarded as excluded from the dextran. A very small peak of included material was also obtained. The fractions constituting both peaks were pooled and dried from the frozen state. The amount of material in the second included pool was very small and was not examined further. A sample of the material in the large excluded pool was hydrolysed with 4N-hydrochloric acid at 100° for 4 hr., glucosamine isolated from the hydrolysate and its specific activity determined. This amino sugar was also isolated from hydrolysates of a trichloroacetic acid extract (5% trichloroacetic acid, heated at 90° for 15 min.) from the material insoluble in ammonia and from the mucopeptide insoluble in both ammonia and hot trichloroacetic acid. The specific activity of the glucosamine hydrochloride in the excluded peak from the ammonia extract was 2840 counts/min./µmole, whereas those of this amino sugar in the other two samples examined were 32.2 and 35.6 respectively. The specific activity of the muramic acid isolated from the hydrolysate of the insoluble mucopeptide was 21.9 counts/min./µmole. A small amount of muramic acid was also present in the excluded peak from the ammonia extract, but was too small in amount for accurate measurement. If, however, all the radioactivity eluted with 10% ethanol from the charcoal-Celite column were due to muramic acid, its specific activity was not less than 3000. Small amounts of the amino acids present in mucopeptide were also to be found in hydrolysates of the excluded peak; anhydrosorbitol and phosphorus were also present. Cell-wall preparations from *S. aureus* strain Oxford that had been incubated in the presence of penicillin (10 µg./ml.) and [³²P]phosphate were also extracted with 0.1N-ammonia under the same conditions as those used in the previous experiment. These extracts were also run through Sephadex G-25 columns and the radioactivity in the eluates was examined (Fig. 4). Two major peaks occurred, and the specific activity and proportion of the phosphate in the pools made from the two peaks is recorded in Table 7: more than half of the radioactivity is contained in the included peak and the specific activity of the phosphorus is 1×10^6 – 2×10^6 counts/min./mg. This value corresponds rather closely to the value for the specific activity of the inorganic [³²P]phosphate initially supplied to the cells. The specific activity of the phosphorus in the excluded peak is lower, about 1×10^5 – 2×10^5 counts/min./mg. Examination of the included peak showed that only

Fig. 3. Fractionation on Sephadex G-25 of material extracted from cell walls with 0.1N-NH₃ in 5 hr. at 20°. The cells of *S. aureus* strain Oxford were first incubated for 30 min. in the usual solution supplemented by 10 µg. of benzylpenicillin/ml. and [U-¹⁴C]glucose. The extract in 0.1N-NH₃ was passed down the column in the same concentration of aq. NH₃ and 6.0 ml. fractions were collected. The abscissa is broken between samples 44 and 73.

Fig. 4. Fractionation of material labelled with ^{32}P extracted from cell walls with aq. NH_3 . The column consisted of Sephadex G-25 and the first peak corresponds in position to that of serum albumin when this was passed down the column. The fractions were of volume 6 ml. and the extract was in 0.1N- NH_3 . The flow of the same solvent was continued.

Table 7. Fractionation of the material removed from walls of organisms treated with penicillin, by alkaline pH

Fractionation of 0.1N- NH_3 extracts of *S. aureus* cell walls on Sephadex G-25 columns (68 cm. $\times$ 3.5 cm.), equilibrated and eluted with 0.1N- NH_3 . Cell walls were isolated from *S. aureus* cells that had been incubated with ^{32}P phosphate and benzylpenicillin in tris-maleate buffer for 30 or 60 min. at 35°. Experimental details are given in the text.

Expt. no.	Time of incubation with penicillin (min.)	Total P ($\mu\text{g.}/\text{mg. dry wt. of cell wall}$)		Sp. activity of P (counts/min./ $\mu\text{g. of P}$)	
		Peak 1	Peak 2	Peak 1	Peak 2
1	30	1.79	0.44	189	2060
2	60	0.91	—	143	—
3	60	2.27	0.41	132	1480
4	60	8.00	1.53	92	6290

inorganic phosphorus could be detected, even when the technique of Marsh (1959) was applied. This technique avoids exposure of the material to acid conditions other than for very brief periods. It seems extremely unlikely that the very active inorganic phosphorus is due to simple adsorption to the wall preparations, in view of the treatment they receive during preparation (i.e. they are washed three times with unlabelled 0.3M-phosphate). Moreover, when the wall preparations made from cells incubated in the presence of penicillin were stood with unlabelled inorganic phosphate solutions at room temperature and pH 7.0, there was little evidence of rapid exchange accounting for more than 10–15% of the total radioactivity. There

was only a slow increase of radioactivity in the soluble phase occurring over a period of 24 hr. Thus it seems probable that the inorganic phosphate present in ammonia extracts is likely to be liberated from organic combination either during the alkaline extraction of the walls or by radiation damage due to the presence of high-specific-activity ^{32}P -phosphate; such damage might occur in the extracts or on the columns. Attempts to find phosphorus in the included region of runs on Sephadex G-25 made with extracts from walls not labelled with ^{32}P phosphate have shown that very small amounts of phosphorus can be found in this region.

DISCUSSION

Perhaps the most significant result recorded above is that deficiency of glycine, glutamic acid and lysine can inhibit the formation of teichoic acid by suspensions of staphylococci. None of these amino acids is a constituent of the ribitol polymer, and it is likely that the quantities in the pool of the organism would be sufficient to maintain the necessary supplies for the biosynthesis of nucleotide coenzymes. Such an observation gives greater credence to the possibility that the penicillins, cycloserine and 5-fluorouracil also have no direct effect on teichoic acid synthesis in the whole cell. Previously it was possible to argue that cycloserine inhibited teichoic acid synthesis by virtue of its known ability to inhibit alanine racemase (Strominger, Ito & Threnn, 1960). This would decrease the supply of the D-alanine attached by ester linkage to the teichoic acid. Likewise, 5-fluorouracil might be supposed to be converted into 5-fluorocytidine, and by forming 5-fluorocDP-ribitol inhibit teichoic acid formation by the same mechanism as that with which fluoroUDP compounds inhibit mucopeptide formation, although equimolar amounts of cytidine fail to reverse the inhibitory effect of 5-fluorouracil on teichoic acid formation. It now appears more reasonable to suppose that the application of any inhibitor of mucopeptide synthesis also leads to some inhibition of teichoic acid formation. Inhibition by penicillin is never complete and varies according to the state of the cells used to make the suspension tested. The teichoic acid that still continues to be formed can be removed by dilute alkali, and in this property differs from the bulk of the teichoic acid formed in the absence of the antibiotic. The total amounts of material removed by alkali from cell walls of staphylococci incubated in the presence and absence of the antibiotic are, however, not very different, although the proportion of the newly formed material is very much greater in the presence of the antibiotic. This observation indicates that, in the presence of

penicillin, there is a continued synthesis of a small amount of teichoic acid that is probably similar in composition to the teichoic acid formed in the absence of the antibiotic but attached to the mucopeptide in a different way. Such a process seems more likely than the formation of a different ribitol phosphate polymer when penicillin is present. Examination of the small amount of material removed by alkali suggests that a moiety of mucopeptide is still attached to it, like that isolated by Ghuyssen & Strominger (1963) after enzymic digestions of walls.

Previous observations (Rogers & Garrett, 1963; Rogers, 1963) showed that 80–85% of the ribitol 'backbone' of teichoic acid could be removed from the mucopeptide by the action of periodate. This result is only explicable if no covalent bonds exist between the oxidized ribitol units and the mucopeptide. This observation and the isolation of a homogeneous teichoic acid with a mucopeptide moiety still attached (Ghuyssen & Strominger, 1963) can only be reconciled by the assumption that not more than one, or at the most two, ribitol units in the teichoic acid are covalently linked to the mucopeptide. Other results (A. J. Garrett & H. J. Rogers, unpublished work) show that the terminal primary alcohol group of the teichoic acid *in situ* in the walls of staphylococci is not substituted. Thus the teichoic acid molecules are likely to be linked to the mucopeptide through their terminal phosphate groups, a result supported by the appearance of phosphate labile to phosphatase when the mucopeptide complex is treated with acids (Strominger & Ghuyssen, 1963). If this is so, then it seems reasonable to suppose that the formation of teichoic acid is in some way dependent on the availability of attachment sites in the mucopeptide. This would be easier to imagine if some of the enzymes concerned in synthesis were fixed to the mucopeptide fibres (Rogers, 1963). Further difficulties are presented, however, by the observations that mucopeptide synthesis in cell-free systems is itself not sensitive to penicillin (Chatterjee & Park, 1964; Meadow *et al.* 1964). It is for this reason that the formation of the bond between teichoic acid, with its moiety of mucopeptide attached, and the fully polymerized mucopeptide becomes an attractive site for the action of the antibiotic. Unfortunately, no specific inhibitor of teichoic acid formation that would not be expected to affect mucopeptide synthesis has been described, and it is therefore not possible to determine whether inhibition of teichoic acid synthesis stops mucopeptide formation. The presence of a small amount of newly synthesized teichoic acid, probably combined with soluble mucopeptide and removable from the walls with alkali, supposedly represents the small amount that can be formed in the absence of available fixation sites. One

would not then expect penicillin to inhibit its formation if the antibiotic acts on the fixation process.

It should, however, be pointed out that more is yet required to be known about the formation of mucopeptide in cell-free systems before it can be concluded with certainty that penicillin does not directly affect some step in mucopeptide synthesis.

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