glucosamine and muramic acid; (b) a mucopeptide containing glycine, muramic acid and glucosamine; (c) a mucopeptide containing muramic acid, glycine, lysine, glutamate and alanine.

4. All the fragments isolated are resistant to the action of lysozyme.

5. The significance of the mucopeptide fragments for the structure of the wall mucopeptide is discussed.

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The Incorporation of Amino Acids into the Cell-Wall Mucopeptide of Staphylococci and the Effect of Antibiotics on the Process

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When washed suspensions of staphylococci in buffered glucose are incubated in the presence of certain single amino acids labelled with ^{14}C , radioactivity is incorporated into the fraction of the cells insoluble in hot trichloroacetic acid (Gale & Folkes, 1953). This process is chloramphenicol-resistant and penicillin-sensitive. Other work (Salton & Horne, 1951; McCarty, 1952a, b; Salton, 1953; Cummins & Harris, 1956) has shown that, after

disruption of Gram-positive bacterial cells, material can be obtained, either by differential centrifuging alone or by a combination of centrifuging and enzyme treatment, which appears, by electron-microscopic examination, to be made up of the cell walls of organisms. Acid hydrolysis and paper chromatography have shown that this material consists preponderantly, in most Gram-positive organisms, of a few amino acids together with

hexoses, glucosamine and another amino sugar, since identified as 3-O- α -carboxyethyl-D-glucosamine, called muramic acid (Strange & Dark, 1956; Kent, 1957; Strange & Kent, 1959). In staphylococci it contains lysine, glycine, glutamate and alanine together with glucosamine and muramic acid. This class of substances from cell walls, which will be referred to as mucopeptides, constitutes a high proportion of the hot trichloroacetic acid-insoluble material from bacteria. The present paper demonstrates that the incorporation observed by Gale & Folkes (1953) into staphylococci under condition 1 (i.e. when only a single amino acid was present) was, in fact, almost exclusively due to the growth of the cell wall. A preliminary account of this work has already appeared (Mandelstam & Rogers, 1958) and similar results with another strain of staphylococci have been reported (Hancock & Park, 1958). Gale, Shepherd & Folkes (1958) have since shown that a high proportion of the incorporation of some amino acids, such as glycine, into the cold trichloroacetic acid-insoluble fraction from partially disrupted preparations of staphylococci (Gale & Folkes, 1955) is also into cell-wall material.

The present paper also reports a study of the inhibitory action of penicillin, bacitracin and aureomycin on the process of mucopeptide synthesis.

METHODS

Organisms. *Staphylococcus aureus* 524 (Rogers, 1953) was grown, with shaking, at 35° in a hydrolysed casein medium supplemented with thiamine (1 μ g./ml.) and nicotinic acid (1 μ g./ml.) (Rogers, 1945). The bacteria were harvested in the exponential phase of growth when the culture contained about 0.8 mg. dry wt. of bacteria/ml. Bacterial concentrations were determined by opacity measurements in a Spekker absorptiometer, with filter 508, and from a calibration curve of opacity against dry wt./ml.

Conditions of cell-wall synthesis. Bacteria were washed twice with 1% (w/v) glucose in 0.05 M-phosphate buffer (prepared from $\text{m-KH}_2\text{PO}_4$ brought to pH 7 with NaOH). They were then suspended in the same solution with the addition of amino acids or ammonium salts (see below) and incubated in 1 l. conical flasks, with shaking, at 35°. The bacterial density was 1.0–1.03 mg. dry wt./ml. and the volume 130 ml. After the incubation period (usually 1 hr.) the contents of the flask were centrifuged, the bacteria were washed once with 40 ml. of 1% (w/v) glucose in buffer and mucopeptide was isolated by the following method, which is essentially that of Cummins & Harris (1956).

Preparation of mucopeptide. Chilled bacterial suspension containing about 150 mg. dry wt. of bacteria was added to Ballotini beads (10 ml.; grade 12, Griffin and George Ltd., London) and shaken in a Mickle disintegrator for 15 min. at 2°. The suspension was filtered through a grade 1 sintered-glass filter to remove the beads and the filter washed twice with water (10 ml.).

The combined filtrate was centrifuged at 10 000 rev./min. for 10 min. in 50 ml. Lusteroid tubes on an MSE

centrifuge model Angle-13 (Measuring and Scientific Equipment Ltd.). The sediment, consisting of cell wall and unbroken cells, was immediately suspended in water (5 ml.) and heated at 100° for 20 min. Omission of this step led to loss of much of the mucopeptide through the action of lytic enzymes (see Mitchell & Moyle, 1957; Richmond, 1959). The preparation was then incubated for 3 hr. at 35° with 1 mg. of trypsin and 1 mg. of ribonuclease in 5 ml. of 0.05 M-phosphate buffer, pH 7.6. The remaining wall material was washed once with 10 ml. of water and resuspended in 5 ml. of 0.5 N-HCl; 1 mg. of crystalline pepsin was added and the preparation was incubated at 35° overnight. The washed mucopeptide was suspended in about 0.5 ml. of water and acetone (5 ml.) was added. The flocculent material was sedimented by centrifuging at low speed, washed with acetone (5 ml.) and ether (5 ml.), dried at room temperature and finally heated at 100° for 1 hr. to remove traces of water. The product was a white powder. The yield of mucopeptide from freshly harvested cells was 10–12.5 mg./100 mg. dry wt. of bacteria.

In those experiments where mucopeptide had been synthesized in the presence of a ^{14}C -labelled amino acid, all washing and digestion procedures were carried out in the presence of the corresponding unlabelled amino acid at a concentration of 2.5 mg./ml.

Isolation of protein. The supernatant obtained after disintegration of the bacteria and removal of cell wall and whole cells was treated with an equal volume of 10% (w/v) trichloroacetic acid. The precipitate was extracted with hot 5% (w/v) trichloroacetic acid and then with acetone, ethanol-ether and ether (see Mandelstam, 1958a). The protein accounted for 30–35% of the dry wt. of the bacteria. In some experiments the whole cells were treated in this manner so that the final product contained both mucopeptide and protein. Such preparations will be referred to as 'mixed trichloroacetic acid precipitates', and accounted for about 50% of the dry wt. of the bacteria.

Composition of mucopeptide. For amino acid determination, 3–4 mg. of mucopeptide was heated at 100° in a sealed tube with 1 ml. of 4 N-HCl for 18 hr. The hydrolysate was dried in a vacuum desiccator containing conc. H_2SO_4 and solid NaOH. To remove traces of HCl the residue was twice dissolved and re-evaporated, about 0.4 ml. of water being used each time. The hydrolysate was dissolved in 0.48 ml. of water and 0.08 ml. amounts were applied (from a graduated 0.1 ml. pipette) to Whatman no. 3 paper as a line about 4 cm. long. Amino acid standards at two concentrations (50 and 100 μ g.) were included on each sheet, and the chromatograms developed with butanol-pyridine-water (6:4:3, by vol.; Jeanes, Wise & Dimler, 1951) for 48 hr. At the end of this time the four amino acids of the mucopeptide were well separated from one another and from the hexosamines. Amino acids were measured by the following modification of the method of Kay, Harris & Entenman (1956). The sheets were thoroughly dried, sprayed with 0.6% ninhydrin in butanol and heated at 50° for 1 hr. The spots were cut out and transferred to test tubes. The colour was eluted with 70% (v/v) ethanol and read in the Spekker absorptiometer with an Ilford Filter 604 (spectrum green) and 1 cm. micro cells. Estimations were carried out at least in duplicate and were reproducible to within $\pm 5\%$.

Glucosamine and muramic acid were determined as described by Perkins & Rogers (1959).

Free amino acid pool. Washed bacteria (10–20 mg. dry wt.) were suspended in 10 ml. of water and heated at 100° for 20 min. to extract free amino acids. Subsequent procedures for separating and estimating the components of the pool were as described by Mandelstam (1958b).

Measurement of radioactivity. Radioactivity of protein, mucopeptide and 'mixed trichloroacetic acid precipitates' was measured on 0.3 cm.² polythene disks at infinite thickness (20 mg./cm.²) with a thin end-window Geiger-Müller counter. Counting was continued until the count was 1000 above the background.

Specific activity of glycine from hydrolysates of protein was determined after isolation of the glycine on paper with water-saturated m-cresol (Mandelstam, 1958a). Estimation of specific activity of glycine in mucopeptide was carried out in the same way except that the paper chromatograms were developed with the butanol-pyridine-water solvent described above.

RESULTS

Biosynthesis of mucopeptide from glucose and glycine in the presence of chloramphenicol

Washed staphylococci were incubated, with shaking, in buffer-glucose solution (see Methods section) containing (per ml.) 200 µg. of [¹⁴C]glycine (specific activity 20 counts/min./µg.) and 50 µg. of chloramphenicol. A control flask without chloramphenicol was incubated at the same time. The opacity of both suspensions increased by 30 % during the experiment.

At the end of 1 hr. 20 ml. samples were taken from each flask and centrifuged. The bacteria were washed and treated with trichloroacetic acid to give 'mixed trichloroacetic acid precipitate'. Mucopeptide and cytoplasmic protein were prepared from the remainder of the suspension and the radioactivity present in each fraction was determined.

It was found (Table 1) that, with the control cells, the specific activity of the mucopeptide was some 20 times as great as that of the protein. Chloramphenicol had little or no effect upon incorporation of radioactivity into mucopeptide but depressed the small incorporation into protein. The specific activity of the 'mixed trichloroacetic acid precipitates' is what would be expected on the

assumption that the mucopeptide constitutes about one-third of the weight of the material in this fraction.

Radioactivity measurements made in this way could be misleading, if, during the incubation, some of the labelled glycine had been converted into other amino acids and these had then been incorporated. For this reason, samples of the fractions were hydrolysed, the glycine was isolated and its specific activity determined.

The results (Table 2) confirm those obtained by measuring the radioactivity of the unhydrolysed materials. The glycine in the protein fraction is 2 % labelled, i.e. its specific activity is 2 % of that of the glycine in the incubation medium. By contrast, the glycine of the mucopeptide is 41 % labelled. Glutamic acid, alanine, lysine and hexosamine separated from the same hydrolysate all had negligible radioactivity and together accounted for about 5 % of the total radioactivity of the mucopeptide. The degree of labelling of the glycine is that which would be expected if the mucopeptide had increased by about 70 % during the incubation. It will be shown (see below) that a net increase of this order does, in fact, occur under these conditions.

It is apparent that chloramphenicol has little effect upon the synthesis of mucopeptide.

Table 1. *Distribution of radioactivity in various cell fractions*

Washed staphylococci were incubated for 1 hr. with glucose (1%, w/v) and [¹⁴C]glycine (200 µg./ml.; specific activity 20 counts/min./µg.) in the presence and in the absence of chloramphenicol (70 µg./ml.). Protein and mucopeptide were isolated after mechanical disintegration of the bacteria (see Methods section).

	Specific activity (counts/min./mg.)	
	Control	With chloram- phenicol
'Mixed trichloroacetic acid ppt.' (protein + mucopeptide)	767	727
Protein	126	62
Mucopeptide	2518	2472

Table 2. *Specific activity of glycine isolated from acid hydrolysates of cell fractions from staphylococci*

Cells were incubated with glucose and [¹⁴C]glycine (see protocol of Table 1), with and without chloramphenicol. In the last column, the specific activity of the glycine from the hydrolysate is expressed as a percentage of that of the glycine added to the incubation medium.

	Glycine (µg.)	Counts/min./µg.	Counts/min./µg. of glycine	Percentage labelling
Without chloramphenicol				
'Mixed trichloroacetic acid ppt.'	242	594	2.5	12.5
Protein	160	67	0.4	2
Mucopeptide	225	1839	8.2	41
With chloramphenicol				
'Mixed trichloroacetic acid ppt.'	176	418	2.4	12
Protein	220	63	0.3	1.5
Mucopeptide	220	1645	7.5	37.5

Requirements for mucopeptide synthesis

The requirements for mucopeptide synthesis were investigated by incubating washed staphylococci for 1 hr. in buffer solution with glucose and one or more of the four amino acids which it normally contains. Owing to the difficulty of handling more than about four samples at a time the results shown in Table 3 have been drawn from several experiments.

Freshly harvested cells contained 9–12 mg. of mucopeptide/100 mg. dry wt. of bacteria and this was increased by a small and variable amount if the cells were incubated with buffer and glucose alone (see Table 3). Addition of DL-alanine, DL-glutamic acid or DL-lysine singly produced a significant increase (up to 25 %) in the amount of mucopeptide. A more marked effect was found with glycine, which uniformly caused a net synthesis of over 50 % in 1 hr. It will be seen that the further addition of glutamic acid or lysine in addition to glycine did not greatly increase the yield of mucopeptides. However, the inclusion of all four amino acids in the incubation medium generally produced an increase of 100–150 %.

Relationship between opacity and synthesis of mucopeptide in washed suspensions

In the type of experiments which we have described, it was found that there was always some increase in the opacity of those bacterial suspensions in which there was a net increase in the mucopeptide content of the cells. Amino acid incorporation (Tables 1 and 2) indicated negligible protein synthesis under these conditions, and analysis of nucleic acid content of the cells, by measuring the absorption at 260 m μ of material extracted by hot trichloroacetic acid (Schneider, 1945), showed that there was an increase of the order of 30 % in nucleic acid content. In spite of this increase in nucleic acid, mucopeptide synthesis was mainly responsible for the increased opacity of the suspensions. The results of a number of experiments were analysed by plotting the actual increase in mucopeptide against the apparent increase in mass, obtained from opacity measurement and the standard calibration curve for the organism. The points lie about a smooth curve, which can be used as a rough indication of mucopeptide synthesis (Fig. 1). The only experiments in which any serious discrepancy was observed between the weight of mucopeptide formed and that expected from opacity measurement were those in which a high concentration (1000 units/ml.) of penicillin was used. In these experiments the increase in mucopeptide was about 50 % less than that expected from the change in opacity (see below).

Table 3. *Conditions for the synthesis of mucopeptide*

Washed staphylococci were incubated for 1 hr. in buffer solution with glucose (1%, w/v) and one or more amino acids at a final concentration of 400 μ g./ml. The bacteria were disintegrated and the mucopeptide was isolated and weighed. Freshly harvested cells contained 9–12 mg. of mucopeptide/100 mg. dry wt. of bacteria.

Additions	Increase in mucopeptide (%)
None	0–10
DL-Lysine	20
DL-Glutamic acid	25
DL-Alanine	20
Glycine	55–80
Glycine + DL-glutamic acid	60
Glycine + DL-lysine	80
Glycine + DL-lysine + DL-glutamic acid + DL-alanine	100–150

Fig. 1. Relationship between extinction and mucopeptide synthesis. Washed staphylococci were incubated for 1 hr. in buffer solution containing 1% (w/v) of glucose and additions as listed below. Samples were taken at the beginning and end of the incubation for isolation of mucopeptide (see Methods section). The increase in mucopeptide has been plotted against the apparent increase in bacterial mass obtained by measurement of extinction. Additions to the glucose-buffer solution: (1) Four amino acids (i.e. glycine, DL-alanine, DL-glutamic acid and DL-lysine, each at 400 μ g./ml.). (2) and (3) As (1). (4) NH_4Cl (1 mg./ml.). (5) Glycine (400 μ g./ml.). (6) As (1) + penicillin (100 units/ml.). (7) As (1) + bacitracin (100 μ g./ml.). (8) None.

Free amino acid content of staphylococci in relation to the synthesis of mucopeptide

The foregoing experiments suggested that glycine might be the limiting factor in mucopeptide synthesis by washed staphylococci incubated with glucose alone. Preliminary experiments showed that the amount of free glycine in the 'pool' from freshly harvested cells was less than that of the other amino acid components of mucopeptide, and also indicated that the glycine might be acting as a source of nitrogen for the synthesis of the other three amino acids.

This conclusion was tested by the following experiment. Washed staphylococci were incubated for 1 hr. in the usual buffer-glucose solution (130 ml.) containing (a) no addition, (b) NH_4Cl (1 mg./ml.) and (c) glycine (400 $\mu\text{g.}/\text{ml.}$). A fourth flask, similar to (a), was set up for the zero-time sample. Duplicate 15 ml. samples were taken from each flask for analysis of the free amino acids. The rest of the bacterial suspension was used for the preparation of mucopeptide.

It was found that, whereas there was no synthesis in the presence of glucose alone, incubation with NH_4Cl or glycine led to a net increase of about 60 % in the mucopeptide content (Table 4).

The analysis of the amino acid pool is given in Table 5. Incubation of the cells with glucose produced a diminution in the concentration of all

four amino acids. This was most noticeable for glutamic acid, which dropped from 3.32 to 0.32 mg./100 mg. of bacteria. Even if this dissipation of pool material had not taken place, it is obvious that, with the exception of glutamic acid, there was not enough of each amino acid at zero time to account for the quantities incorporated into the mucopeptide during the experiment. A comparison of the zero-time column and the final column of Table 5 shows that this deficit is most manifest for glycine and hexosamine, which together constitute 60 % of the mucopeptide.

When NH_4Cl was present, not only was there synthesis of mucopeptide (7.6 mg./100 mg. of bacteria) but the concentrations of the free amino acids (lysine excepted) and hexosamine increased. The results with glycine were broadly similar to those obtained with NH_4Cl . The main differences were that the concentrations of glutamic acid and hexosamine were lower, and that of glycine was, as might be expected, a great deal higher.

Composition of mucopeptide

Analysis of mucopeptide isolated from cells harvested at about 0.8 mg. dry wt. of bacteria/ml. of culture was carried out by the techniques described in the Methods section.

The main amino acid components were lysine, glycine, glutamic acid and alanine (no attempt has been made to determine the optical configuration of these amino acids). In addition glucosamine and muramic acid were found and, if it is assumed that they were in the *N*-acetylated form, over 90 % of the mucopeptide preparation could be accounted for. The remainder consisted of small and variable amounts of other amino acids. Valine, serine and leucine (or isoleucine) were each present to the extent of 0.5–2 %. In addition, similar amounts of aspartic acid were found in some of the preparations, as well as occasional traces of phenyl-alanine.

Table 4. *Mucopetide content of staphylococci*

Incubation was for 1 hr. in buffer-glucose solution with additions: (a) none; (b) NH_4Cl (1 mg./ml.); (c) glycine (400 $\mu\text{g.}/\text{ml.}$).

	Mucopetide (mg./100 mg. of bacteria)
Zero-time sample	12.2
(a) No addition	12.0
(b) NH_4Cl	19.8
(c) Glycine	20.2

Table 5. *Free amino acids during synthesis of mucopeptide*

Washed staphylococci were incubated for 1 hr. with buffer-glucose solution with additions: (a) none; (b) NH_4Cl (1 mg./ml.); (c) glycine (400 $\mu\text{g.}/\text{ml.}$). Samples for free amino acid analysis were taken before and after incubation. It has been assumed that the hexosamine was present as glucosamine. The last column shows the amount of each amino acid required for the mucopeptide synthesized in flask (c) during the experiment (7.6 mg./100 mg. of bacteria). The corresponding quantities for (b) are 5 % lower. The values have been calculated assuming the following composition of mucopeptide: lysine, 7 %; glutamic acid, 12 %; glycine, 20 %; alanine, 14 %; hexosamine, 43 %.

	Amino acid ($\mu\text{g.}/100$ mg. dry wt. of bacteria)				Required for mucopeptide
	Zero-time	(a)	(b)	(c)	
Lysine	170	120	130	130	560
Glutamic acid	3320	320	5730	1620	960
Glycine	40	20	80	1920	1600
Alanine	970	750	1420	1270	1120
Hexosamine	—	—	200	70	3400
Total	4500	1210	7560	5010	7640

Table 6. *Composition of mucopeptide isolated from five different cultures of staphylococci*

Cells were harvested during exponential growth. Values are given as percentages, and those for glucosamine and muramic acid refer to the *N*-acetylated amino sugars.

	1	2	3	4	5	Mean
Glutamic acid	11.6	14.5	11.7	12.7	10.5	12.2
Glycine	17.5	23.1	21.1	20.5	18.6	20.1
Alanine	12.5	16.0	14.2	13.8	12.6	13.8
Lysine	6.2	8.5	8.0	6.9	6.3	7.2
Glucosamine	15.0	—	—	16.9	18.7	16.9
Muramic acid	31.0	—	—	24.0	24.4	26.6
Total (four amino acids)	47.8	62.1	55.0	53.9	48.0	53.3
Total (two amino sugars)	46.0	—	—	40.9	43.1	43.5

The detailed quantitative analysis of five such preparations is given in Table 6. Variations exceeding that of the analytical error were encountered from one batch of mucopeptide to another, and the values in the table have been chosen to cover the extremes of the range. The four main amino acids together constituted 44–62 % of the preparation, and the two amino sugars 40–47 %. In spite of the variation, the total accounted for by these six substances was invariably 90–95 % of the particular sample. The final column of the table gives the mean values, and from these the molar ratios have been calculated, glutamic acid being taken as unity. The values are:

glutamic acid : glycine : alanine :

1 : 3.3 : 1.9 :

lysine : glucosamine : muramic acid

0.6 : 1.0 : 1.1

Analyses were also carried out on mucopeptide synthesized in the presence of (a) NH_4Cl , (b) glycine, (c) glycine and the other three amino acids (added in DL form). The values fall within the range shown in Table 6.

Effect of penicillin, bacitracin and aureomycin upon synthesis of mucopeptide

The experiments already described showed that staphylococci in the presence of glucose and the four amino acids provide a convenient system for studying mucopeptide synthesis in isolation from protein synthesis. The effect of penicillin upon mucopeptide synthesis was investigated by incubating washed cells in buffer-glucose solution containing glycine, DL-alanine, DL-glutamic acid and DL-lysine (each at a final concentration of 400 $\mu\text{g./ml.}$) and benzyl penicillin (100 and 1000 units/ml.). These high concentrations of penicillin were necessary because, in this strain of staphylococci, penicillinase formation can be induced by penicillin. In addition, chloramphenicol (70 $\mu\text{g./ml.}$) was added to prevent formation of the enzyme. The control flask contained the amino acids and chloramphenicol.

The flasks were shaken for 1 hr. and the muco-

Fig. 2. Effect of penicillin upon synthesis of mucopeptide. Washed staphylococci were incubated in buffer solution containing 1 % (w/v) of glucose and glycine, DL-alanine, DL-glutamic acid and DL-lysine (each at 400 $\mu\text{g./ml.}$). Chloramphenicol (70 $\mu\text{g./ml.}$) was added to prevent induced formation of penicillinase. Increase in mucopeptide was obtained from measurements of extinction and the calibration curve shown in Fig. 1. O, Control; ●, penicillin (100 units/ml.); △, penicillin (1000 units/ml.).

peptide was isolated. Also, during the incubation, the extinction of the suspensions was measured at intervals.

The synthesis of the mucopeptide in the control flask appeared to be linear after the first 15 min. (Fig. 2). In the presence of 100 units of penicillin/ml. a lowered rate was detectable from the start, and synthesis had more or less ended after 45 min. This concentration of penicillin appeared to be sufficient to obtain a maximal effect, since a tenfold increase in the amount of penicillin did not greatly increase the degree of inhibition. In subsequent experiments (see below) penicillin was used at 500 units/ml.

In Table 7 the increase in mucopeptide indicated by the extinction at the end of 1 hr. has been compared with the actual increase determined by weighing. The two methods of estimation agree very well for the control culture and that containing 100 units of penicillin/ml., but the opacity increase obtained with 1000 units/ml. indicates the synthesis of about twice as much mucopeptide as was actually recovered at the end of the experiment.

The effect of bacitracin (100 $\mu\text{g./ml.}$) was examined in the same way, by incubating cells with the amino acids in glucose-buffer solution; chloramphenicol was omitted. For the first 15 min., mucopeptide synthesis, as measured by extinction, was the same in the presence and in the absence of antibiotic. After this, the rate declined progressively so that at the end of an hour the amount of mucopeptide synthesized was about one-half of that obtained in the control culture (Fig. 3). In a similar experiment, in which the mucopeptide was isolated and weighed, it was found that synthesis was inhibited 58 % by bacitracin.

The effect of aureomycin (100 $\mu\text{g./ml.}$) was also investigated. At this concentration no mucopeptide synthesis was detectable.

Composition of mucopeptide synthesized in the presence of penicillin

Analysis of the mucopeptide estimated after incubation of cells with four amino acids and penicillin (see above) gave a low value for alanine (11.0 %, compared with 12.7 % for the control) and suggested that the antibiotic might interfere not only with the amount of mucopeptide synthesized but also with its composition. Any such effect is obviously obscured by the fact that the major part (about 70 %) of the sample analysed consists of normal material which was present at the beginning of the experiment. The newly synthesized mucopeptide might be expected to show a divergence from the normal value about two to three times as great as that apparent from the crude analytical figures.

In an attempt to obtain some definite evidence on this point, experiments of the following type were done with labelled amino acids. Washed staphylococci were incubated as before in flasks containing buffer-glucose with chloramphenicol and 400 $\mu\text{g./ml.}$ of each of the four amino acids previously used. In the first flask the alanine was labelled (14 500 counts/min./ μmole). Another flask had the same additions, except that the glutamic acid was labelled instead of the alanine (15 140 counts/min./ μmole). After incubation for 1 hr. mucopeptide was isolated from both suspensions and the radioactivity measured. The activity (counts/min./mg.) in the alanine- and glutamate-labelled mucopeptides was 307 and 179, giving a

ratio of 1.72. Correction of this value for the difference in molar specific activity of the two amino acids gives a ratio of alanine:glutamic acid 1.8:1. Within experimental error this is the ratio found by chemical analysis for normal mucopeptide. Mucopeptide, however, isolated from a similar pair of cultures containing penicillin (500 units/ml.) gave a ratio of 1.4:1. In another experiment the ratio was even lower (0.9:1). The

Table 7. *Effect of penicillin on synthesis of mucopeptide*

Washed staphylococci were incubated in buffer-glucose with glycine, DL-alanine, DL-glutamic acid and DL-lysine (each at 400 $\mu\text{g./ml.}$) in the presence and in the absence of penicillin. Chloramphenicol (70 $\mu\text{g./ml.}$) was present to prevent induced formation of penicillinase. The amount of mucopeptide synthesized after incubation for 1 hr. has been estimated from increase in extinction of the bacterial suspension and also by weighing.

	Increase in mucopeptide (mg./100 mg. of bacteria)	
	Extinction	Weight
Control	9.0	8.5
Penicillin (100 units/ml.)	5.5	4.8
Penicillin (1000 units/ml.)	4.3	2.0

Fig. 3. Effect of bacitracin and aureomycin upon synthesis of mucopeptide. Washed staphylococci were incubated in 1 % (w/v) glucose-buffer solution containing glycine, DL-alanine, DL-glutamic acid and DL-lysine (each at 400 $\mu\text{g./ml.}$). Increase in mucopeptide was obtained from measurement of extinction and the calibration curve shown in Fig. 1. $\blacktriangle$, Control; $\circ$, bacitracin (100 $\mu\text{g./ml.}$); $\bullet$, aureomycin (100 $\mu\text{g./ml.}$).

analyses with labelled amino acids therefore confirm those carried out by chemical methods and show that there is a fall in the ratio of alanine to glutamic acid in mucopeptide formed in the presence of penicillin.

Preliminary results suggested that there might also be a decrease in the ratio of glycine to glutamic acid, but these observations have not been pursued.

DISCUSSION

If it is assumed that the amino acids extractable from the cells by hot water can be used directly for cell-wall synthesis, and either have the right optical configuration or can be converted rapidly by the action of the appropriate racemases, there is sufficient glutamic acid and alanine present to allow the synthesis of 7–8 mg. of mucopeptide/100 mg. of cells. The amount of extractable lysine is enough for about one-third of this amount of mucopeptide, but there is little or no available glycine or hexosamine. This deficiency of glycine in the 'pool' would alone be sufficient to explain, qualitatively, the greater incorporation of glycine and the more active cell-wall synthesis which occurs when only this amino acid is added. The quantitative experiments reported here show that, with our strain of staphylococci, glycine can also serve as a source of nitrogen for the synthesis of other amino acids, as, in fact, can the NH_4^+ ion. Whether or not the carbon of glycine can also be used for amino acid synthesis cannot be determined from these experiments, owing to the presence of large amounts of unlabelled carbon fragments arising from the glucose. That the breakdown of formed protein does not give sufficient amino acids to affect the results is due to the slow rate at which this process occurs, i.e. 1–2 %/hr. (J. Mandelstam, unpublished observations).

Although the formation of both proteins and mucopeptide must involve peptide-bond synthesis, these two processes can be readily dissociated. If the cells are incubated either with any one of the amino acids present in the cell wall together with glucose, or with a full complement of amino acids, glucose and chloramphenicol, no significant protein synthesis occurs, whereas cell-wall mucopeptide is formed. Contrariwise, if penicillin is added, mucopeptide formation is inhibited; but protein synthesis can continue (Gale & Folkes, 1953; Strominger, 1957). Park (1958) has reported similar results. Aureomycin stops the formation of proteins, mucopeptide and nucleic acid.

It is to be noted that penicillin does not stop the biosynthesis of mucopeptide immediately; synthesis continues for about 45 min. at a reduced rate before it ceases. This long period of delay would seem unlikely to be due either to slow penetration

of the antibiotic to the synthesizing sites or to its destruction by small amounts of penicillinase in the cells. For the former possibility the period is too long and if the latter were true one might have expected an initial period of inhibition followed by an acceleration in synthesis. It is also difficult to understand the period of delay in terms of the exhaustion of precursors of the process, if mucopeptide 'biosynthesis' is to be explained in terms of a single invariable pathway. If, however, two independent pathways leading either to more than one compound or to one compound of variable composition are presupposed, then the kinetics of action of the antibiotic are comprehensible. One step in one pathway may be inhibited immediately, thus stopping synthesis by this route, whilst synthesis can still proceed by the second route, either until the supply of its intermediates is exhausted or until metabolic imbalance of some sort becomes too great for the cell. Such a hypothesis is supported by our finding that the mucopeptide synthesized in the presence of penicillin has a different composition from that in the control cells. Owing to the difficulty of finding solvents for the mucopeptide which are not likely to hydrolyse covalent bonds, homogeneity tests have so far been impossible.

Strominger & Park (1957) have suggested that the mechanism of penicillin action is to prevent further incorporation of the peptide, isolated by Park (1952*a, b, c*), into cell wall. If this hypothesis were wholly correct then one might expect synthesis of mucopeptide to stop almost immediately on the addition of penicillin. This does not occur, and a search for peptides of the type isolated by Park in the cells of our strain of staphylococci, inhibited by penicillin, has been unsuccessful (unpublished observations), so that, if present, their concentration must be very low.

An alternative to the present suggestion of a dual mechanism for normal mucopeptide synthesis might be that the presence of penicillin causes a different mucopeptide to be synthesized by way of a partially resistant pathway. This process may be slower than the normal and may cease when its intermediates are exhausted.

One result of this study has been to make unnecessary, at least in this system, the intellectually difficult hypothesis of exchange of amino acids in formed protein. Because radioactive amino acids were incorporated into staphylococcal 'mixed protein' under conditions where no net increase in protein could be demonstrated, Gale & Folkes (1953) and Gale (1957) proposed that the amino acids in the already formed protein exchanged with the exogenous supply. It has now been shown that the incorporation into whole cells which occurred under these authors' conditions was entirely due to

synthesis of cell-wall mucopeptide. Whether or not other substances, such as teichoic acid (Baddiley, Buchanan & Carss, 1958; Armstrong, Baddiley, Buchanan & Carss, 1958), which are associated with isolated cell walls in some organisms, are also synthesized under these conditions has not been tested. The method of mucopeptide preparation selected here is likely to remove them from the cell wall, and also the values for the radioactive content of the 'mixed trichloroacetic acid precipitates' agree with those for the isolated mucopeptide. Again, the treatment with hot 5% trichloroacetic acid in the preparation of mixed precipitates would certainly be expected to remove any teichoic acid.

SUMMARY

1. Staphylococci incubated in the presence of ^{14}C -labelled glycine, glutamate, alanine or lysine, either singly or together, incorporate these almost exclusively into the cell-wall mucopeptide.

2. This incorporation can be entirely accounted for by the increase in mass of cell-wall mucopeptide. This process is resistant to chloramphenicol.

3. The amino acids can be replaced by glucose and ammonium chloride with the strain of *Staphylococcus aureus* used.

4. The synthesis of cell-wall mucopeptide is inhibited by penicillin and bacitracin, neither of which inhibits protein synthesis.

5. Some evidence is presented which suggests that the small amount of mucopeptide synthesized in the presence of penicillin has an abnormal composition.

6. The relation of these observations to the mechanism of penicillin action and to a hypothesis concerned with protein synthesis is discussed.

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Observations on the Composition and Metabolism of the Nitrogen-Fixing Root Nodules of *Myrica*

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In an earlier study of the fate of labelled nitrogen fixed by the root nodules of *Alnus* (Leaf, Gardner & Bond, 1958) the greatest abundances of ^{15}N were found in the carbamyl group of citrulline and the amino group of glutamic acid. This was taken to indicate that an early product of fixation was possibly ammonia, and that though the pattern of

nitrogen metabolism in *Alnus* differs in other respects from that of the leguminous plants and other nitrogen-fixing organisms, their systems for nitrogen fixation had this much in common. This paper describes similar work undertaken with the root nodules of *Myrica*, which have also been shown to fix nitrogen (Bond, 1951, 1958).