Iodinated Vancomycin and Mucopeptide Biosynthesis by Cell-Free Preparations from *Micrococcus lysodeikticus*

By CLAUDETTE BORDET AND H. R. PERKINS

National Institute for Medical Research, Mill Hill, London N.W.7, U.K.

(Received 9 June 1970)

A particulate preparation from *Micrococcus lysodeikticus* was used to synthesize cell-wall mucopeptide. Radioactive iodinated vancomycin became attached to the preparation simultaneously with a complete inhibition of mucopeptide synthesis. After mucopeptide synthesis had occurred in the absence of antibiotic, the preparation took up more vancomycin, suggesting that new binding sites terminating in acyl-D-alanyl-D-alanine had been produced. The mucopeptide product was divided into a soluble and an insoluble portion, both sensitive to lysozyme. The soluble portion did not combine with vancomycin and hence had presumably lost its terminal D-alanine residues, either by transpeptidation or because of carboxypeptidase action. The synthesis of both portions was unaffected by the presence of penicillin, but the insoluble part showed increased affinity for vancomycin, thus indicating that penicillin had caused conservation of D-alanyl-D-alanine termini.

Vancomycin is an antibiotic that is known to interfere with the synthesis of cell-wall mucopeptide in sensitive bacteria (Reynolds, 1961, 1966; Jordan, 1961; Best & Durham, 1964) and cell-free preparations synthesizing mucopeptide are sensitive to vancomycin (Anderson, Matsushashi, Haskin & Strominger, 1965; Anderson, Meadow, Haskin & Strominger, 1966).

Sensitive cells or their isolated cell walls are known to absorb considerable quantities of vancomycin (Best & Durham, 1965) and a small proportion of the antibiotic, labelled by the introduction of radioactive iodine, was shown to be present in preparations of the cytoplasmic membrane (Perkins & Nieto, 1970). Vancomycin and ristocetin are also known to combine with the nucleotide precursors of mucopeptide that terminate in D-alanyl-D-alanine (Chatterjee & Perkins, 1966) and in fact the sequence required for specific combination is acyl-D-alanyl-D-alanine, or a close structural analogue thereof (Perkins, 1969; Perkins & Nieto, 1969). The inference that specific combination with a key sequence of mucopeptide precursors is involved in the action of these drugs seems inescapable. Complex-formation could occur at any time in the biosynthetic process when a precursor with a peptide chain terminating in D-alanyl-D-alanine was accessible to vancomycin and Best, Grastie & McConnell (1970) have shown that vancomycin has a greater affinity for the cell walls of *Micrococcus lysodeikticus* than for the nucleotide precursor. We have attempted, by using radioactive iodinated

vancomycin in conjunction with a cell-free system for mucopeptide biosynthesis, to discover at what stage vancomycin might, by its continued attachment to intermediates, interfere in the process.

METHODS

Antibiotics. Vancomycin hydrochloride was given by Eli Lilly and Co. Ltd., Basingstoke, Hants., U.K. Iodinated vancomycin, with or without ^{125}I , was prepared as described by Perkins & Nieto (1970). Sodium benzylpenicillin was obtained from Glaxo Laboratories Ltd., Greenford, Middx., U.K. Monosodium benzylpenicilloate was synthesized as described earlier (Perkins, 1969).

Paper chromatography. Whatman no. 3 and 3MM papers were used for preparative purposes after being washed with M-ammonium acetate and with water. The following solvents were used: solvent A, isobutyric acid-aq. 0.5 M- NH_3 (5:3, v/v); solvent B, ethanol-M-ammonium acetate (5:2, v/v).

Preparation of the mucopeptide precursor. The mucopeptide precursor UDP-MurNAc*-L-Ala-D-Glu-L-Lys-D-Ala-D-Ala (referred to below as UDP-MurNAc-pentapeptide) was prepared by accumulation in a medium containing EDTA, which acts by deprivation of bivalent cations, as described by Garrett (1969a,b). *M. lysodeikticus* N.C.T.C. 2665 was grown at 35°C in Hedley-Wright broth containing 1% of glucose. Cells (about 1 g dry wt.) from an exponentially growing culture were resuspended (1.5-2.0 mg dry wt./ml) in a 50 mM-sodium phosphate-1 mM-EDTA (sodium salt) buffer, pH 7.0, containing 1% of glucose, 0.005% of chloramphenicol and 0.02% of each of

* Abbreviation: MurNAc (in peptide sequences), N-acetylmuramyl.

the following amino acids: DL-alanine, DL-glutamic acid, L-lysine and glycine (Garrett, 1969a,b). They were incubated with continued shaking for 1 h at 35°C, collected by centrifugation, and extracted in ice-cold 25% (w/v) trichloroacetic acid (50 ml). The soluble material was freed from trichloroacetic acid by extraction with ether, applied to a column (100 cm × 2 cm) of Sephadex G-25 and eluted with water on a fraction collector. Samples were hydrolysed in 0.1 M-HCl in a boiling-water bath for 5 min and total *N*-acetylhexosamine was measured by the method of Reissig, Strominger & Leloir (1955). Fractions containing *N*-acetyl-amino sugar were pooled and the nucleotides were separated by chromatography on Whatman no. 3 paper in solvents *A* and *B*. The yield of UDP-MurNAc-pentapeptide was about 15 μmol. UDP-MurNAc-L-Ala-D-[¹⁴C]Glu-L-Lys-D-Ala-D-Ala was prepared in the same way after addition of D-[¹⁴C]glutamic acid (50 μCi) to the medium containing EDTA. The radioactive material was subjected to paper electrophoresis in 0.25 M-formic acid. The areas corresponding to free glutamic acid and to the mucopeptide precursor UDP-MurNAc-pentapeptide were cut out and their radioactivities measured; there was no radioactivity in the area corresponding to free glutamic acid and the specific radioactivity of the labelled precursor was 8.3×10^4 c.p.m./μmol.

Particulate enzyme. The particulate enzyme of *M. lysodeikticus* was prepared by the method of Matsushashi, Dietrich & Strominger (1967). Cells were grown in Hedley-Wright broth containing 1% of glucose in shaken flasks at 35°C. Cultures growing exponentially were harvested and the cells were washed with 20 mM-tris-HCl buffer, pH 8.0. They were then ground with a pre-cooled pestle and mortar for 10 min at 2°C with 3 times their wet weight of levigated alumina (Norton, Welwyn Garden City, Herts., U.K.). Cells-alumina mixture (1 g) was suspended in 4 ml of 50 mM-tris-HCl buffer, pH 7.5, containing 0.1 mM-MgCl₂ and 1 mM-2-mercaptoethanol and centrifuged twice at 15000g. The fraction sedimenting between 15000g and 105000g was collected, washed twice and finally dispersed in the buffer to a final protein concentration of 7 mg/ml. Protein was measured by the method of Lowry, Rosebrough, Farr & Randall (1951).

Incubations. A typical incubation mixture contained 9 nmol (7500 counts/10 min) of ¹⁴C-labelled UDP-MurNAc-pentapeptide, 13 nmol of UDP-*N*-acetylglucosamine, 17.5 μmol of tris-HCl buffer, pH 8.6, 6 μmol of MgCl₂ and 70 μl of membrane preparation (490 μg of membrane protein) in a total volume of 0.14 ml. Incubations were carried out for various times at 37°C, stopped by heating at 100°C for 1 min and subjected to chromatography on Whatman 3MM paper in solvent *A* for 15 h. When required, the mixtures after inactivation were centrifuged for 15 min at 15000g. The pellet and the supernatant fraction were then separately subjected to paper chromatography. The chromatograms were cut into 1 cm strips, which were counted for radioactivity in a Packard Tri-Carb liquid-scintillation spectrometer. The scintillation fluid consisted of 2,5-diphenyloxazole (10 g) and 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene (0.25 g) in 2.5 litres of toluene. The efficiency of measurement of radioactivity was about 40%.

In experiments with [¹²⁵I]iodinated vancomycin, the antibiotic was added to the incubation mixture either before or after incubation. In the latter case the mixture

was then incubated for a further 10 min at 37°C. Chromatograms were prepared as before, and 1 cm strips were cut out. Radioactivity was measured with a Packard Auto-Gamma Spectrometer (efficiency 39%).

RESULTS

In each experiment an incubation under the standard conditions, with ¹⁴C-labelled UDP-MurNAc-pentapeptide as substrate, was carried out to check the activity of the membrane fraction. After paper chromatography in solvent *A* for 15 h the following products were obtained: *R_F* 0, mucopeptide; *R_F* 0.24, residual UDP-MurNAc-pentapeptide; *R_F* 0.35, a non-identified product (X); this may have been UDP-MurNAc-tetrapeptide obtained by the action of a carboxypeptidase, but it was not positively identified; *R_F* 0.9, the 'lipid intermediate' of mucopeptide biosynthesis (Tipper & Strominger, 1965). No incorporation into mucopeptide occurred in the absence of added UDP-*N*-acetylglucosamine.

Incubation in the presence of vancomycin inhibits mucopeptide synthesis, partially or completely, depending on the concentration of antibiotic. To specify the role of the antibiotic in this inhibition, two types of experiments were performed with radioactively labelled or unlabelled iodinated vancomycin.

Unlabelled iodinated vancomycin. Incubations were carried out with ¹⁴C-labelled UDP-MurNAc-pentapeptide and unlabelled iodinated vancomycin (107 μg/ml, 1 mol/mol of substrate). The radioactivity of the different products obtained after paper chromatography is shown for a typical experiment in Fig. 1; the biosynthesis of the mucopeptide was 90–100% inhibited in different experiments. There was some radioactivity in the initial nucleotide, in the product X and in the lipid intermediate. When less iodinated vancomycin was added inhibition of mucopeptide synthesis decreased; 21 μg/ml (0.2 mol/mol of substrate) gave 15% inhibition and 15 μg/ml (0.15 mol/mol of substrate) no inhibition.

[¹²⁵I]Iodinated vancomycin. Incubations were carried out with unlabelled UDP-MurNAc-pentapeptide and radioactive iodinated vancomycin (1 mol/mol of substrate). After paper chromatography there was some radioactivity in different parts of the chromatogram. Comparison of the radioactivity distribution after incubation with radioactively labelled and unlabelled iodinated vancomycin (Table 1) shows that ¹²⁵I was present at the origin of the chromatogram even when mucopeptide synthesis was completely inhibited. The presence of [¹²⁵I]iodinated vancomycin at a position corresponding to *R_F* 0.24 indicates that the compound between nucleotide and iodinated vancomycin possessed in this solvent system about the same

Fig. 1. Chromatography of radioactive products from UDP-MurNAc-pentapeptide labelled with ^{14}C -glutamic acid. Incubations were carried out as described in the Methods section with (---) or without (—) addition of 1 mol of unlabelled vancomycin/mol of substrate. After incubation, the mixtures were subjected to paper chromatography in solvent A. Then the chromatograms were cut into strips 1 cm wide and radioactivity was measured.

Table 1. Incubations with iodinated vancomycin

Membrane preparations from *M. lysodeikticus* were incubated with mucopeptide precursors as described in the Methods section. Two incubations were simultaneously carried out, one with unlabelled nucleotide precursor and 107 $\mu\text{g}/\text{ml}$ of [^{125}I]iodinated vancomycin (1 mol/mol of substrate), the other with ^{14}C -labelled nucleotide precursor and 107 $\mu\text{g}/\text{ml}$ of unlabelled iodinated vancomycin. The products were chromatographed in solvent A. Strips (1 cm wide) were cut out and used for measurement of radioactivity. The values are calculated from the specific radioactivity of the substances used, and represent the incorporation into each sample.

	Incorporation (nmol)	
	[^{125}I]Iodinated vancomycin. Unlabelled precursor	Unlabelled iodinated vancomycin. ^{14}C -labelled precursor
Mucopeptide ($R_f 0$)	0.03	0
Nucleotide ($R_f 0.24$)	4.5	5.1†
Product X ($R_f 0.35$)	0	1.05
Lipid intermediate* ($R_f 0.9$)		0.55

* There was some indication of slight incorporation of [^{125}I]iodinated vancomycin into the lipid intermediate. It could not be measured accurately, however, because of overlap with free ^{125}I -labelled vancomycin.

† i.e. unchanged precursor.

mobility as the free nucleotide (Perkins & Nieto, 1970). Free iodinated vancomycin appeared as a long spot from $R_f 0.44$ to $R_f 0.85$.

The presence of [^{125}I]iodinated vancomycin at the origin of the chromatogram when mucopeptide synthesis was inhibited suggests a combination between the antibiotic and a substance, $R_f 0$, existing in the original membrane preparation.

When [^{125}I]iodinated vancomycin was added to the mixture after incubation, the amount of ^{125}I at the origin of the chromatogram increased with the time of incubation (Table 2). These results suggest a combination between the antibiotic and the newly formed mucopeptide, since very little iodinated vancomycin was found in the membrane when no mucopeptide synthesis occurred.

To test whether a combination between iodinated vancomycin and the membrane preparation before incubation could inhibit mucopeptide synthesis, the membrane preparation was mixed at neutral pH with labelled or unlabelled iodinated vancomycin and then washed to remove free antibiotic (Table 3). After incubation of ^{14}C -labelled precursor with the membrane that had been pre-treated with unlabelled iodinated vancomycin, mucopeptide synthesis was greatly inhibited (90%) (Table 3, assay 1).

However, at the pH value of the incubation, pH 8, there was some dissociation of the membrane-iodinated-vancomycin complex and exchange reactions occurred between the different acceptors of the antibiotic; thus, after incubation of unlabelled precursor with the membrane that had been pre-treated with [^{125}I]iodinated vancomycin (Table 3, assay 2), we found 2.26 nmol of free iodinated vancomycin, although it had been carefully removed

Table 2. *Combination between iodinated vancomycin and newly formed mucopeptide*

Two parallel experiments were performed simultaneously as described in Table 1 and the Methods section except that [^{125}I]iodinated vancomycin was added before incubation (time 0) and after incubation with unlabelled precursor for the times indicated.

Time of incubation (min)	[^{125}I]Iodinated vancomycin or newly formed ^{14}C -labelled mucopeptide at origin of the chromatogram (pmol)	
	^{125}I	^{14}C
0	30	0
60	64	1740
120	100	2400
150	120	2640
150*	30	—

* Without precursor.

before incubation, and 1.27 nmol of nucleotide-linked iodinated vancomycin. Some dissociation of the membrane-iodinated-vancomycin complex also occurred in solvent *A*, even without added nucleotide.

Study of the mucopeptide formed during incubation. To specify the nature of the newly-formed mucopeptide, an incubation was carried out under the standard conditions for 2 h at 37°C with 9 nmol of ^{14}C -labelled UDP-MurNAc-pentapeptide and after inactivation by boiling for 1 min the mixture was centrifuged at 15000 *g* for 15 min. Then the pellet and the supernatant fraction were separately subjected to paper chromatography in solvent *A* for 16 h. Two different products were found remaining at the origin of the chromatogram; one was soluble and in the supernatant, the other was insoluble and present in the pellet. After 2 h of incubation the soluble polymer constituted 52–62% of the total mucopeptide synthesized in five experiments. The products from the supernatant and from the pellet were both digested by lysozyme.

It was possible that there was initially only one polymer present and that partial solubilization in the incubation buffers had occurred. A similar incubation with ^{14}C -labelled precursor was carried out and after centrifugation the pellet was carefully washed three times with 100 μl of incubation buffers and every supernatant fraction was counted for radioactivity. The first supernatant contained 58% of the total radioactivity found at the origin of the chromatograms, the next two contained 9.5 and 9.0% respectively and the fourth only 1%. The final residue contained 22%. Thus the bulk of the soluble material was clearly not produced by slow release of the insoluble material.

Table 3. *Mucopeptide synthesis by a membrane fraction previously mixed with iodinated vancomycin*

The membrane-iodinated-vancomycin complexes were prepared by mixing iodinated vancomycin (9 nmol) in a volume of 5 μl and 70 μl of membrane preparation from *M. lysodeikticus* containing 880 μg of protein. After centrifugation at 105000 *g* for 10 min the pellets were washed three times to remove the free vancomycin and were then dispersed in 70 μl of fresh supernatant prepared by centrifuging a similar volume of membrane preparation. Membrane preparation washed in the same way but without added vancomycin served as a control. Two experiments were performed: membrane fraction was pre-treated with unlabelled iodinated vancomycin and then incubated with ^{14}C -labelled UDP-MurNAc-pentapeptide (assay 1); membrane fraction was pre-treated with [^{125}I]iodinated vancomycin and then incubated with unlabelled UDP-MurNAc-pentapeptide (assay 2). Incubations were carried out at 37°C for 2 h. Assay 1 shows the decrease in mucopeptide biosynthesis caused by previous treatment of the membrane preparation with iodinated vancomycin, and assay 2 is a parallel experiment to show where the adsorbed iodinated vancomycin was to be found after the second incubation with the complete biosynthetic system.

	Control ^{14}C -labelled penta- peptide (nmol)	Assay 1 ^{14}C -labelled penta- peptide (nmol)	Assay 2 ^{125}I -labelled vancomycin (nmol)
Mucopeptide (R_f 0)	1.95	0.21	0.06
Nucleotide (R_f 0.24)	3.9	6.05	1.27
Free iodinated vancomycin (R_f 0.44–0.85)			2.26

The time-course of the biosynthesis of the soluble and insoluble polymers is shown in Fig. 2. At early times most of the product (70%) was insoluble but as mucopeptide synthesis proceeded the proportion decreased until at 120 min only 46% was insoluble.

It is known that the synthesis of mucopeptide by particulate preparations is not sensitive to low concentrations of penicillin, the effect of the drug being more probably involved with prevention of cross-linking (Tipper & Strominger, 1965). Nevertheless, in our previous experiments synthesis of one of the mucopeptide fractions could have been inhibited by penicillin with a concomitant increase in incorporation into the other fraction. To test whether the presence of penicillin affected the synthesis of either the soluble or the insoluble portion of the mucopeptide, incubations were performed in the absence and in the presence of this antibiotic. In the presence of penicillin, both soluble and insoluble polymers were synthesized in about the same proportions as in its absence, although the amount of soluble polymer slightly decreased (Table 4). Both polymers formed

in the presence of penicillin were digested by lysozyme (Table 4). Since the lysozyme digestion preceded the separation of the soluble and insoluble polymers, the residual radioactivity found as 'soluble polymer' after digestion with lysozyme might represent original soluble mucopeptide that survived digestion, or partially digested insoluble polymer. The overall digestion of both polymers was nevertheless considerable.

Fig. 2. Time-course of biosynthesis of mucopeptide fractions. UDP-MurNAc-pentapeptide labelled with [^{14}C] glutamic acid (45 nmol), UDP-*N*-acetylglucosamine (65 nmol), tris-HCl buffer, pH 8.6 (87.5 μmol), MgCl_2 (30 μmol) and membrane protein (2.5 mg) in a total volume of 0.7 ml were incubated at 37°C. At the times indicated, samples (0.12 ml) were removed, inactivated by boiling for 1 min and centrifuged at 15000 *g* for 15 min. Pellets and supernatant fractions were separately subjected to paper chromatography in solvent *A*. The origins of the chromatograms were cut out and radioactivity was measured. Δ, Soluble mucopeptide; ○, insoluble mucopeptide.

Uptake of iodinated vancomycin by the soluble and insoluble mucopeptides. Particulate preparation was incubated for 2 h under the standard conditions with UDP-MurNAc-pentapeptide. After incubation [^{125}I]iodinated vancomycin was added to the mixture, which was then centrifuged. The pellet and the supernatant fraction were subjected to paper chromatography in solvent *A* and products at the origin of the chromatogram were cut out and counted for radioactivity. There was no radioactivity in the supernatant fraction; only the pellet was radioactive. Thus, vancomycin combined with the insoluble mucopeptide but not with the soluble one. Other incubations in the presence and absence of penicillin were carried out, iodinated vancomycin being added after incubation. The mixtures contained either ^{14}C -labelled precursor to measure mucopeptide formation or unlabelled nucleotide precursor, in which case [^{125}I]iodinated vancomycin was added at the end of the incubation. The $^{125}\text{I}/^{14}\text{C}$ incorporation ratio indicated the proportion of peptide chains of the mucopeptide bound to a molecule of iodinated vancomycin (Table 5). Vancomycin did not combine with the soluble mucopeptide formed by incubation with or without penicillin. Since the amounts of soluble and insoluble mucopeptides are about equal, the $^{125}\text{I}/^{14}\text{C}$ ratios were about twice as great in the insoluble mucopeptide as in the unfractionated product (Table 5).

In all these experiments, iodinated vancomycin combined with the insoluble mucopeptide but the $^{125}\text{I}/^{14}\text{C}$ ratio increased threefold in the presence of penicillin. To test whether this result was due to an association between the two antibiotics, an incubation was carried out in the presence of sodium benzylpenicilloate. This substance, although not antibiotically active, apparently combines more easily with vancomycin than does benzylpenicillin itself (Perkins, 1969). However, it had no effect on

Table 4. Formation of soluble and insoluble polymers in the presence of penicillin

Membrane preparations from *M. lysodeikticus* were incubated with ^{14}C -labelled UDP-MurNAc-pentapeptide as described in the Methods section for 2 h at 37°C with the additions indicated. Lysozyme digestion was performed after inactivation (1 min of boiling) by adding 40 μg of lysozyme and re-incubating for 20 min at 37°C. Then the mixtures were centrifuged at 15000 *g* for 15 min, and pellets and supernatants were separately subjected to paper chromatography in solvent *A*. The origins of the chromatograms were cut out and radioactivity was measured. The radioactivity is expressed in counts/10 min.

Additions	Incorporation into mucopeptide			
	Expt. 1		Expt. 2	
	Soluble polymer	Insoluble polymer	Soluble polymer	Insoluble polymer
None (control)	813	765	644	553
Benzylpenicillin (10 $\mu\text{g}/\text{ml}$)	719	724	562	574
Benzylpenicillin (10 $\mu\text{g}/\text{ml}$), then lysozyme digestion	112	47		

Table 5. *Ratio of iodinated vancomycin to newly-formed mucopeptide*

Membrane preparations from *M. lysodeikticus* were incubated with mucopeptide precursors as described in the Methods section with additions as indicated. In each experiment two parallel assays were carried out: (1) incubations with ^{14}C -labelled precursor to measure the amount of mucopeptide formed, as determined by the radioactivity of the chromatogram origin; (2) incubations with unlabelled precursor and inactivated by boiling before addition of [^{125}I]iodinated vancomycin to measure the uptake of antibiotic. In Experiment 2 the mucopeptide was separated into soluble and insoluble fractions as described for Table 4. The soluble mucopeptide did not retain any vancomycin. The values refer to the amount of radioactivity recovered from the origins of chromatograms (mucopeptide fraction).

Additions	Total mucopeptide			Insoluble mucopeptide		
	Iodinated vancomycin (nmol)	Muco-peptide (nmol)	$^{14}\text{C}/^{125}\text{I}$ molar ratio	Iodinated vancomycin (nmol)	Muco-peptide (nmol)	$^{14}\text{C}/^{125}\text{I}$ molar ratio
Experiment 1						
None	0.1	2.4	24			
Benzylpenicillin (10 $\mu\text{g}/\text{ml}$)	0.24	1.92	8			
Sodium benzylpenicilloate (10 $\mu\text{g}/\text{ml}$)	0.08	2.24	28			
Experiment 2						
None				0.06	0.92	15
Benzylpenicillin (10 $\mu\text{g}/\text{ml}$)				0.18	0.87	5

mucopeptide synthesis or vancomycin uptake (Table 5). Thus, the threefold increase in the amount of [^{125}I]iodinated vancomycin present at the origin of the chromatogram after incubation in the presence of penicillin was not due to an association of the two antibiotics but to a combination between vancomycin and the insoluble mucopeptide.

DISCUSSION

Vancomycin caused inhibition of the synthesis of mucopeptide by particulate preparations from *Staphylococcus aureus*, *M. lysodeikticus*, *Escherichia coli* and *Salmonella newington* (Anderson, Matsuhashi, Haskin & Strominger, 1965, 1967; Izaki, Matsuhashi & Strominger, 1968). The formation of the lipid intermediate (Higashi, Strominger & Sweeley, 1967) was not inhibited, sometimes even being enhanced by the presence of the antibiotic (Matsuhashi *et al.* 1967). The present experiments with radioactive iodinated vancomycin show that antibiotic was bound to the membrane preparation from *M. lysodeikticus* that was used to study mucopeptide biosynthesis. Further, an active membrane preparation that was pre-treated with iodinated vancomycin and thoroughly washed showed 90% inhibition of mucopeptide synthesis. Vancomycin is known to combine with mucopeptide precursor nucleotides (Chatterjee & Perkins, 1966) and with other sequences terminating in acyl-D-alanyl-D-alanine (Perkins, 1969). According to the generally accepted scheme of mucopeptide biosynthesis in

cell-free preparations, a disaccharide-pentapeptide unit is formed while still attached to the lipid intermediate and then transferred to an acceptor, which is presumed to be a piece of pre-formed mucopeptide (Anderson *et al.* 1966; Wise & Park, 1965; Tipper & Strominger, 1965; Siewert & Strominger, 1967). If such an acceptor retained its terminal D-alanyl-D-alanine sequence, then vancomycin could bind to it. The attachment of the bulky molecule of antibiotic could then conceivably prevent the acceptor from functioning as such, leading to inhibition of mucopeptide synthesis and accumulation of lipid intermediate.

In whole cells treated with iodinated vancomycin, much of the antibiotic bound to the cells appeared in the wall fraction (Perkins & Nieto, 1970), but with longer contact an increasing proportion was recovered in the membrane fraction. Protoplasts of *M. lysodeikticus*, formed by the action of lysozyme in the presence of sucrose and Mg^{2+} , took up far less vancomycin into the membrane fraction (Perkins & Nieto, 1970). However, those results do not permit a direct comparison with the uptake into the membrane fraction, produced by grinding with alumina, that was used in the present experiments. It is notable that iodinated vancomycin, although not removed from the membrane preparation by washing, was released during incubation with precursor nucleotide. This parallels the release of vancomycin bound to cell walls by the addition of specific peptide (Perkins & Nieto, 1970). The fact that some free iodinated vancomycin appeared on the chromatogram probably corresponded to the disappearance

of terminal D-alanyl-D-alanine originally present in the added precursor and to some dissociation of the complex in the chromatographic solvent.

The separation of the biosynthetic mucopeptide into soluble and insoluble portions recalls the results of Araki, Shimada & Ito (1966) and Izaki *et al.* (1968). The soluble products were formed by particulate preparations from *E. coli*, but only in the presence of added penicillin. Izaki *et al.* (1968) presented evidence suggesting that their 'spreading product' consisted of un-cross-linked mucopeptide. A similar 'spreading product' was produced when whole cells (but not particulate preparations) of *Staph. aureus* incorporated glycine into mucopeptide in the presence of penicillin (Matsuhashi *et al.* 1967). In the present work the soluble product appeared without addition of penicillin; the fact that it was destroyed by lysozyme implies that it was indeed mucopeptide. Since it trapped no vancomycin it apparently had no residual side chains terminated by D-alanyl-D-alanine, so that it could either be cross-linked or have lost its terminal D-alanine residues by the action of a carboxypeptidase. Anderson *et al.* (1966) were unable to demonstrate the formation of any cross-linked mucopeptide by particulate preparations from *M. lysodeikticus*, and we have no direct evidence of the occurrence of cross-linking. The insoluble mucopeptide that remained in the washed membrane appeared to have some intact D-alanyl-D-alanine termini, since its synthesis was accompanied by increased ability to bind vancomycin. The proportion of vancomycin bound, however, represented only one molecule for every 15–25 peptide chains of newly synthesized mucopeptide. In the presence of penicillin the proportion increased to one molecule for every 5–8 chains, suggesting that the presence of penicillin had prevented either transpeptidation or removal of C-terminal D-alanine residues by a carboxypeptidase. Either of these conclusions would support previous suggestions for the effect of penicillin action (Wise & Park, 1965; Tipper & Strominger, 1965; Izaki *et al.* 1968).

It is theoretically possible that vancomycin could attach itself to the pentapeptide chains of precursors that were still bound in the lipid intermediate. We obtained little evidence of vancomycin in the position occupied by lipid intermediate on chromato-

grams, but it remains possible that lipid intermediate with bound iodinated vancomycin would move to a position obscured by the free antibiotic.

C.B. is on leave from the Laboratoire de Chimie Biologique, Université de Lyon, Lyon, France.

REFERENCES

- Anderson, J. S., Matsuhashi, M., Haskin, M. A. & Strominger, J. L. (1965). *Proc. natn. Acad. Sci. U.S.A.* **53**, 881.
- Anderson, J. S., Matsuhashi, M., Haskin, M. A. & Strominger, J. L. (1967). *J. biol. Chem.* **242**, 3180.
- Anderson, J. S., Meadow, P. M., Haskin, M. A. & Strominger, J. L. (1966). *Archs Biochem. Biophys.* **116**, 487.
- Araki, Y., Shimada, A. & Ito, E. (1966). *Biochem. biophys. Res. Commun.* **23**, 518.
- Best, G. K. & Durham, N. N. (1964). *Archs Biochem. Biophys.* **105**, 120.
- Best, G. K. & Durham, N. N. (1965). *Archs Biochem. Biophys.* **111**, 685.
- Best, G. K., Grastie, M. K. & McConnell, R. D. (1970). *J. Bact.* **102**, 476.
- Chatterjee, A. N. & Perkins, H. R. (1966). *Biochem. biophys. Res. Commun.* **24**, 489.
- Garrett, A. J. (1969a). *Biochem. J.* **112**, 15P.
- Garrett, A. J. (1969b). *Biochem. J.* **115**, 419.
- Higashi, Y., Strominger, J. L. & Sweeley, C. C. (1967). *Proc. natn. Acad. Sci. U.S.A.* **57**, 1878.
- Izaki, K., Matsuhashi, M. & Strominger, J. L. (1968). *J. biol. Chem.* **243**, 3180.
- Jordan, D. C. (1961). *Biochem. biophys. Res. Commun.* **6**, 167.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
- Matsuhashi, M., Dietrich, C. P. & Strominger, J. L. (1967). *J. biol. Chem.* **242**, 3191.
- Perkins, H. R. (1969). *Biochem. J.* **111**, 195.
- Perkins, H. R. & Nieto, M. (1969). *Abstr. FEBS 6th Meet., Madrid*, no. 679.
- Perkins, H. R. & Nieto, M. (1970). *Biochem. J.* **116**, 83.
- Reissig, J. L., Strominger, J. L. & Leloir, L. F. (1955). *J. biol. Chem.* **217**, 959.
- Reynolds, P. E. (1961). *Biochim. biophys. Acta*, **52**, 403.
- Reynolds, P. E. (1966). *Symp. Soc. gen. Microbiol.* **16**, 47.
- Siewert, G. & Strominger, J. L. (1967). *Proc. natn. Acad. Sci. U.S.A.* **57**, 767.
- Tipper, D. J. & Strominger, J. L. (1965). *Proc. natn. Acad. Sci. U.S.A.* **54**, 1133.
- Wise, E. M. & Park, J. T. (1965). *Proc. natn. Acad. Sci. U.S.A.* **54**, 75.