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Reversal by a Specific Peptide (Diacetyl-αγ-L-diaminobutyryl-D-alanyl-D-alanine) of Vancomycin Inhibition in Intact Bacteria and Cell-Free Preparations

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Vancomycin inhibited the growth of *Bacillus megaterium*, *Staphylococcus aureus* and *Micrococcus lysodeikticus*, and in cell-free preparations from *B. megaterium* it inhibited the formation of mucopeptide and enhanced the accumulation of the lipid intermediate in the biosynthetic pathway. All these inhibitory processes were reversed by the presence of a synthetic peptide analogous to un-cross-linked mucopeptide side chains, namely diacetyl-L-diaminobutyryl-D-alanyl-D-alanine. A considerable amount of vancomycin was found in recovering cells, whether recovery was caused by peptide or took place naturally because a low initial concentration of antibiotic was used. In cell-free preparations pre-treated with vancomycin, continued inhibition of mucopeptide synthesis depended on the presence of cell-wall material. This inhibition was also reversible by added peptide.

Vancomycin inhibits the growth of Gram-positive bacteria and interferes with the biosynthesis of cell-wall mucopeptide (peptidoglycan) both in whole cells (Reynolds, 1961, 1966; Jordan, 1961) and in cell-free preparations (Anderson *et al.*, 1965, 1967; Reynolds, 1971*a*,*b*). It binds to the cell walls of sensitive bacteria (Best & Durham, 1965; Jordan, 1965) and also to mucopeptide precursor nucleotides (Chatterjee & Perkins, 1966) and other peptides terminating in D-Ala-D-Ala (Perkins, 1969) or related structures, in which both the terminal residues are a D-amino acid or glycine (Nieto & Perkins, 1971*b*). Since the binding of specific peptide by vancomycin is related to its antibiotic activity, it has been proposed that complex-formation may be the basis for the antibiotic action (Perkins, 1969; Perkins & Nieto, 1970; Nieto & Perkins, 1971*a*). If this were true, soluble peptide that binds vancomycin should compete with the binding sites in living cells and hence reverse inhibition caused by the antibiotic. Our experiments show that reversal by a specific peptide of vancomycin inhibition can be obtained both in whole cells and in cell-free preparations from broken whole cells or from 'reconditioned' protoplasts (Reynolds, 1971*a*,*b*). The use of radioactively labelled iodovancomycin (Perkins & Nieto, 1970) has enabled us to follow the fate of the antibiotic in these experiments. The results obtained provide a rational basis for the reversal of vancomycin inhibition of cell-free synthesis of mucopeptide brought about by the addition of cell-wall preparations from *Micrococcus lysodeikticus* (Sinha & Neuhaus, 1968).

Methods

Antibiotics

Vancomycin hydrochloride (Vancocin HCl) was kindly given by Eli Lilly and Co. Ltd., Basingstoke, Hants., U.K. Iodovancomycin (^{125}I-labelled) was prepared as described by Nieto & Perkins (1971*a*).

Growth of bacteria

For experiments in which whole bacteria were treated with antibiotic, *Bacillus megaterium* KM, *Micrococcus lysodeikticus* (N.C.T.C. 2665) and *Staphylococcus aureus* H were grown in conical flasks on a reciprocal shaker at 35°C in CY medium (Novick, 1963) supplemented with 1% glucose. Under these conditions and at a bacterial density of 0.2–0.3 mg dry wt./ml the minimum inhibitory concentration for *B. megaterium* was 2–4 μg of vancomycin hydrochloride or iodovancomycin/ml, and 8–10 μg/ml for *M. lysodeikticus* and *S. aureus*.

For experiments with cell-free preparations from broken whole cells or with protoplast membranes, *B. megaterium* was grown and the preparations were made as described by Reynolds (1971*a*,*b*).

Precursor

UDP-*N*-acetylmuramyl-L-alanyl-D-isoglutamyl-(L)-*meso*-diaminopimelyl-(L)-D-alanyl-D-alanine [where (L) before diaminopimelic acid indicates that

the amino group bound is at the L-centre, and (L) after it indicates that the bound carboxyl group is at the same centre] was prepared, radioactively labelled with diamino[^{3}H]pimelic acid, as described by Reynolds (1971*a*), and is referred to as UDP-*N*-acetylmuramylpentapeptide. Two preparations were used, (1) specific radioactivity 58 Ci/mol and (2) specific radioactivity 9 Ci/mol.

Incubation mixture

For biosynthesis of mucopeptide in cell-free systems, the incubation mixture contained tris–HCl buffer, pH 7.8 (0.1 M), UDP-*N*-acetylmuramyl-pentapeptide (0.4 mM), UDP-*N*-acetylglucosamine (0.4 mM), $MgCl_2$ (15 mM) and particulate enzyme (2–8 mg of protein/ml) in a final volume of 28 μl. The temperature was 23°C. Protein was measured by the method of Lowry *et al.* (1951) with dry bovine serum albumin as standard.

Measurement of incorporation

After incubation the samples were inactivated in a boiling-water bath for 1 min and then chromatographed on paper for 16 h in isobutyric acid–1 M-NH_3 (5:3, v/v). The areas of the paper occupied by the lipid intermediates were cut out and counted directly for radioactivity in a liquid-scintillation counter, and the radioactivity associated with the mucopeptide at the origin of the chromatogram was eluted in 6 M-HCl at 37°C for 24 h and, after evaporation of the HCl *in vacuo*, was redissolved in water before counting for radioactivity in scintillation fluid (Reynolds, 1971*a*) in a Phillips liquid-scintillation analyser.

[^{125}I]Iodovancomycin

The radioactivity of 1 ml samples was measured in a Panax well counter (counting efficiency for ^{125}I, 42.5%).

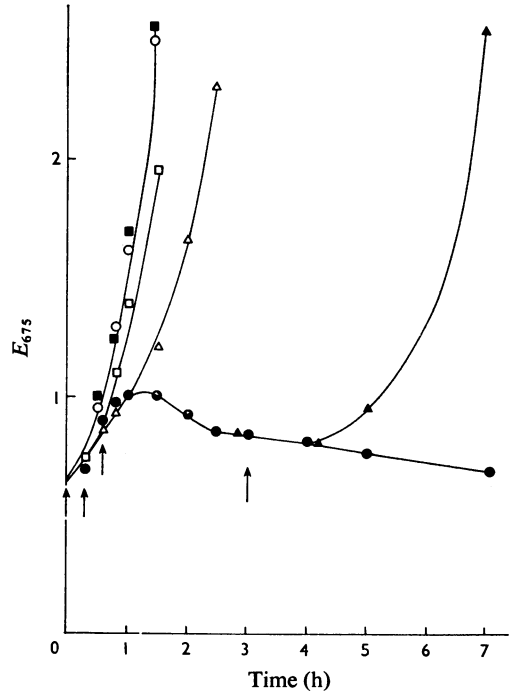

Fig. 1. *Reversal by specific peptide of the inhibition by iodovancomycin of B. megaterium*

Exponentially growing bacteria (0.3 mg dry wt./ml) were incubated in growth medium with no additions (○); prior addition of iodovancomycin (5 μg/ml) (●); prior addition of iodovancomycin (5 μg/ml) together with peptide (diacetyl-L-diaminobutyryl-D-alanyl-D-alanine) (0.1 μmol/ml) (■); prior addition of iodovancomycin (5 μg/ml) and addition of peptide (0.1 μmol/ml) at 15 min (□), 30 min (△), and 3 h (▲). Arrows indicate the times of peptide addition. This figure is reproduced from Perkins & Nieto (1972) by kind permission of Springer-Verlag.

Results

Reversal of growth inhibition

Cultures of *B. megaterium*, *M. lysodeikticus* and *S. aureus* growing exponentially were diluted into pre-warmed medium containing sufficient iodovancomycin to cause inhibition of growth. The peptide diacetyl-L-diaminobutyryl-D-alanyl-D-alanine was added either before the bacteria or later. This particular peptide was used as a matter of convenience but any related peptide with a comparable affinity for vancomycin (Nieto & Perkins, 1971*b*) would have served equally well. The inhibition of growth of *B. megaterium* caused by iodovancomycin was prevented by the simultaneous presence of peptide (molar ratio peptide/antibiotic, 38) and reversed by its subsequent addition (Fig. 1). The molar ratio of peptide to antibiotic was chosen in the light of the observed values of the affinity of vancomycin for the cell walls of a particular species compared with its affinity for soluble peptide (Nieto, 1971). Addition of peptide after 15 min was effective almost immediately but cells that were exposed to the antibiotic for a longer period before the addition of peptide took longer to recommence growth. After recovery all cultures were examined microscopically to check for contamination, but none was found. However, the cells growing after 3 h contact with iodovancomycin showed,

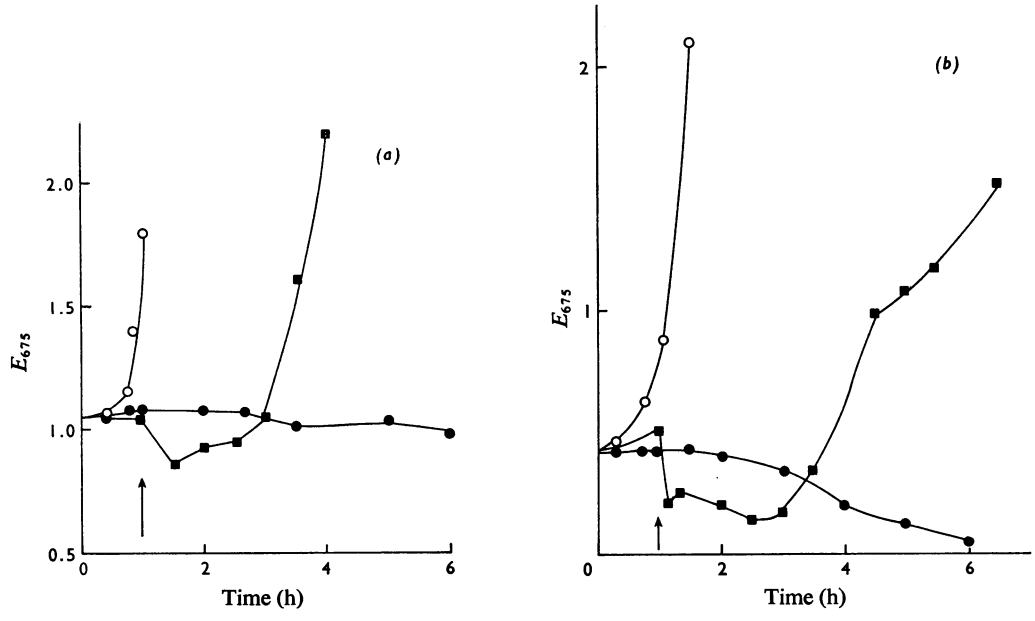

Fig. 2. *Reversal by specific peptide of the inhibition by iodovancomycin of S. aureus H and M. lysodeikticus*

Exponentially growing *S. aureus* H (*a*) or *M. lysodeikticus* (*b*) (0.2–0.5mg dry wt./ml) were incubated in growth medium with no additions (○), prior addition of iodovancomycin (15μg/ml) alone (●) or with addition after 1h of diacetyl-L-diaminobutyryl-D-alanyl-D-alanine (0.4μmol/ml) (■). The arrows indicate the time of peptide addition.

Table 1. *Distribution of iodovancomycin between B. megaterium and the medium during inhibition of growth and reversal of the inhibition by specific peptide*

Duplicate cultures of exponentially growing bacteria were incubated with [^{125}I]iodovancomycin (5μg/ml) for 30min under normal growth conditions. Then to one flask peptide (0.1mM) was added and at the stated intervals samples were taken and centrifuged and the radioactivity retained by the cells was measured. Samples were also taken from the flask without added peptide; here the proportion of total radioactivity found in the cells remained constant until the onset of lysis about 50min after the antibiotic had been added. The concentration of iodovancomycin in the cells was calculated from their observed radioactivity, the known specific radioactivity of the antibiotic and the concentration of cells deduced from E_{675}.

Time after addition of peptide (min)	Concn. of bacteria (mg dry wt./ml)	Percentage of total radioactivity found in cells	Iodovancomycin in cells (μg/mg dry wt. of bacteria)
Before addition	0.19	24	6.2
Immediately after addition	0.19	12	3.3
15	0.19	14	3.6
60	0.19	14	3.7
90	0.20	18	4.4
150	0.32	32	5.0

during the first few generations after the reversal of inhibition, somewhat unusual morphology, perhaps indicative of altered cell division.

Growing cells of *M. lysodeikticus* and *S. aureus* were treated similarly, except that peptide (peptide/antibiotic molar ratio, 50) was added only after 1h

of incubation with the antibiotic. In each case reversal of inhibition occurred, with rapid growth recommencing 2h after the addition of peptide (Fig. 2). The decrease in extinction observed on addition of the peptide coincided with aggregation of the bacteria and did not appear to be caused by lysis. When rapid growth was resumed the cells of *S. aureus* disaggregated, but those of *M. lysodeikticus* did not, so that readings of extinction became unreliable. These difficulties probably account for the apparent irregularity of resumed growth observed with *M. lysodeikticus*. As with *B. megaterium*, morphological variability was observed during the early stages of resumption of growth.

Amount of iodovancomycin present in inhibited and recovering cells

After addition of peptide. By using [^{125}I]iodovancomycin, the amount of antibiotic that remained in recovering cells could be measured. In one experiment cells growing exponentially were incubated with iodovancomycin for 30min and samples were taken before the addition of peptide, immediately afterwards and at intervals until the cells began to grow again (Table 1). The proportion of radioactivity retained by the cells was 24% at first and this decreased to 12% immediately after addition of peptide. A low value was maintained until growth began again, when the proportion once more began to increase, reaching 18% just before rapid growth commenced and 32% when the cell concentration had nearly doubled. Inhibited cells at the point of resumption of growth (90min; Table 1) still contained 4.4μg (2.3nmol) of iodovancomycin/mg of bacteria. From the results obtained by Luria (1960) the average mass of a cell of *B. megaterium* is 2pg dry wt. and hence the antibiotic remaining in recovering cells would represent about 3×10^6 molecules per cell. Presumably these molecules that remain must be in parts of the cell that are not involved in the inhibitory action of vancomycin. The amount of vancomycin removed by peptide addition (about 2.5–2.9μg/mg dry wt. of bacteria; Table 1) must be sufficient to allow the relevant synthetic processes to start again, but in this experiment, although vancomycin has been removed from sensitive sites, some has no doubt been set free from insensitive sites also, so that we cannot tell how much needs to be released from sensitive sites before growth can recommence.

The fate of adsorbed vancomycin in experiments like those of Fig. 1 was also followed. The experimental design was similar to that used in the recovery experiments with peptide added after non-radioactive antibiotic. Samples were taken immediately before addition of peptide and also after growth had resumed and the cell concentration had doubled. The radioactivities of each sample, and of the cells and supernatant solution in it, were then measured. The results show that although the cultures after doubling in the presence of added peptide had taken up more of the available antibiotic, the final concentration of iodovancomycin in the recovering cells was about 40% less than in inhibited cells (Table 2). Bacteria that were never inhibited because the peptide was present before the addition of antibiotic con-

Table 2. *Distribution of iodovancomycin between B. megaterium and the medium during inhibition of growth and after reversal of the inhibition by specific peptide*

A culture of exponentially growing bacteria (0.19mg dry wt./ml) was treated as described in Fig. 1. [^{125}I]Iodovancomycin had a specific radioactivity of 4850d.p.m./μg (9.05×10^6 d.p.m./μmol). At the times indicated peptide (0.1mM) was added and after various intervals growth resumed (see Fig. 1). Samples were taken immediately before addition of peptide (inhibited cells) and again about one generation after resumption of growth, centrifuged and the radioactivity of the cells and the medium was measured. When peptide was added at 3h the cells grew overnight before the final sample was taken. Peptide addition at 0min means that the bacteria were added to medium already containing both iodovancomycin and peptide, and no growth inhibition occurred. The concentration of iodovancomycin in the cells was calculated as in Table 1.

Time of peptide addition (min)	Sample	Concn. of bacteria (mg dry wt./ml)	Percentage of total radioactivity found in cells	Iodovancomycin in cells (μg/mg dry wt. of bacteria)
0	After 3s	0.19	3	0.86
	After 90min	0.52	16	1.6
15	Inhibited	0.19	23	7.4
	Growth resumed	0.46	33	4.0
30	Inhibited	0.19	21	6.6
	Growth resumed	0.48	33	3.8
180	Inhibited	0.19	22	6.8
	Growth resumed	2.5	42	0.95

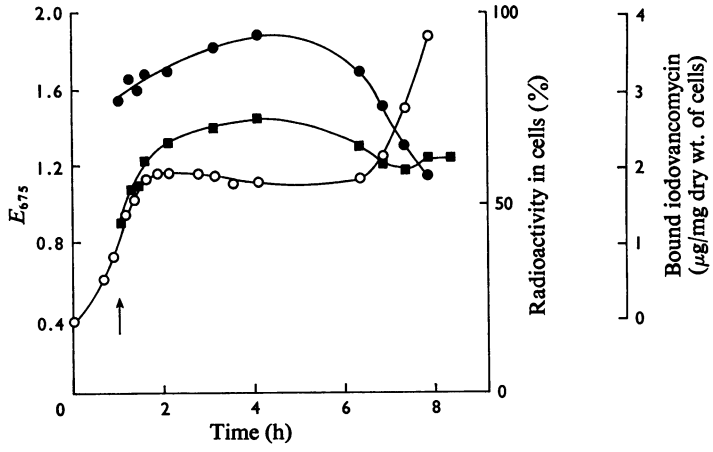

Fig. 3. *Distribution of iodovancomycin in cultures of B. megaterium recovering from inhibition*

Exponentially growing bacteria (0.47mg dry wt./ml) were treated with [^{125}I]iodovancomycin (3μg/ml) at the time indicated by the arrow. Samples taken at intervals were immediately centrifuged and the proportion of the total radioactivity present in the cells was measured. E_{675} of the culture (○); percentage of the total radioactivity present in the cells (■); concentration of iodovancomycin in the cells (μg of antibiotic/mg dry wt.) (●).

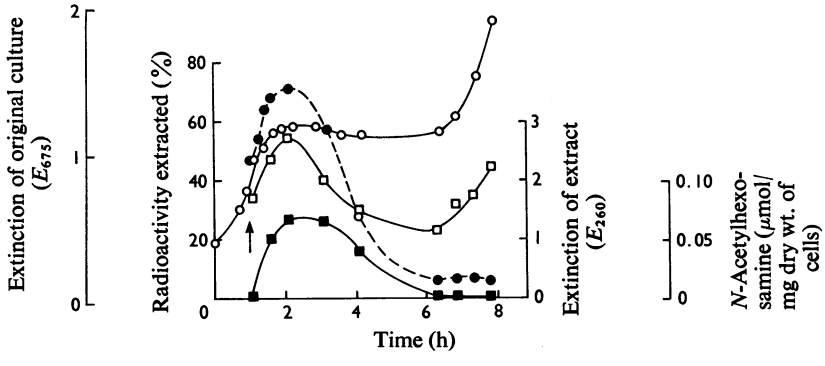

Fig. 4. *Extraction into buffer of iodovancomycin adsorbed by B. megaterium during inhibition and recovery of growth*

The experiment is the same as in Fig. 3. Cells from samples taken at intervals were recovered by centrifuging, resuspended in the same volume of 0.01 M-sodium phosphate buffer, pH 7, and incubated at 36°C overnight. The suspension was centrifuged and the proportion of the radioactivity present in the supernatant solution was measured (●). The E_{260} (□) and the *N*-acetylhexosamine content after brief acid hydrolysis (■) of the supernatant solutions and the E_{675} of the parent culture (○) are also shown. Addition of antibiotic is indicated by the arrow.

tained 23% of the radioactivity found in inhibited cells. Thus cells that had undergone no growth inhibition, because peptide added simultaneously with vancomycin competed for the available antibiotic, contained after 90min 1×10^6 molecules of iodovancomycin per cell calculated as before. Evidently these molecules must have been bound in such a way that sufficient sensitive sites were free to allow unimpeded growth.

Spontaneous recovery. This problem was also studied by observations on cells recovering from inhibition by lower concentrations of vancomycin (6–8μg/mg dry wt. of bacteria). *B. megaterium* growing exponentially at about 0.5mg dry wt./ml was treated with 3μg of radioactive iodovancomycin/ml and samples taken at various times were used for measurement of the radioactivity in the cells and in the medium (Fig. 3). Growth finally ceased at about

1h after the addition of antibiotic and did not recommence for another 5h. The proportion of the total antibiotic found in the cells increased from about 45% immediately after addition to 60% when growth had ceased and rose still further to 70% during a period of bacteriostasis lasting 2h. Before growth recommenced, however, some iodovancomycin was excreted into the medium, and this trend continued during the first hour or so of rapid growth. Although excretion occurred, the amount of antibiotic bound to the cells at the resumption of growth (3.25 μg/mg dry wt.) was no less than when growth had originally come to a halt (3.2 μg/mg dry wt.). This value is less than in the inhibited cells in Tables 1 and 2 and is about the same as in the recovering cells of those experiments, in which a much higher dose of antibiotic was used. It is clear that only a small proportion of the adsorbed vancomycin can be attached at sensitive sites, and that during spontaneous recovery either these sites are freed of vancomycin or new sites are created to take their place.

Excretion of adsorbed iodovancomycin was also observed when cells were removed from the culture, collected by centrifuging, resuspended in the same volume of 0.01 M-sodium phosphate buffer, pH 7, and incubated overnight at 36°C (Fig. 4). No lysis of the cells occurred, since the E_{675} of the suspension remained unaltered. The amount of antibiotic excreted by the cells was related to the stage at which they were removed from the original inhibited culture. Thus maximum excretion (70% of the adsorbed iodovancomycin) occurred from cells washed and resuspended in buffer at the time when growth of the culture had completely ceased. During the period of bacteriostasis and eventual recovery, the proportion of the antibiotic that was excreted when the cells were resuspended in buffer decreased to less than 10% of the amount adsorbed. The excretion of iodovancomycin into buffer was accompanied by the excretion of material absorbing at 260nm (the amount of vancomycin was not sufficient to account for more than an insignificant proportion of the extinction). Measurement of acid-labile *N*-acetylhexosamine (Reissig *et al.*, 1955) showed that this material was excreted in parallel with the antibiotic. The maximum amount of acid-labile *N*-acetylhexosamine excreted was about 70nmol/mg dry wt. of bacteria. The corresponding amount of iodovancomycin extracted was about 1.7nmol/mg dry wt. of bacteria.

Experiments with *B. megaterium* cultures treated with sufficient iodovancomycin to preclude recovery showed that the antibiotic was still excreted into the medium. The pattern of excretion was similar to that shown in Fig. 3 and cells resuspended in buffer again showed maximum excretion of antibiotic during the early stages of inhibition, accompanied by the excretion of material absorbing at 260nm. These results show that in the first 1–2h after inhibition the antibiotic can be removed from the cells by incubation in buffer, although in cells remaining in growth medium in the continued presence of antibiotic no iodovancomycin was released from the cells during this period (Fig. 3). Some antibiotic was, however, released from the cells in the growth medium during the period preceding resumption of growth, at a time when release during subsequent incubation in buffer had declined.

Reversal of inhibition in cell-free systems

The vancomycin-sensitive synthesis of mucopeptide by broken cell preparations and membranes from 'reconditioned' protoplasts of *B. megaterium* has been demonstrated (Reynolds, 1971*a*,*b*). When a membrane preparation from 'reconditioned' protoplasts was incubated with vancomycin, the simultaneous presence of diacetyl-L-diaminobutyryl-D-alanyl-D-alanine completely prevented the 90% inhibition of mucopeptide synthesis caused by the antibiotic (Fig. 5). If peptide was added to an inhibited preparation after 1h, biosynthesis of mucopeptide was resumed at the same rate at which it was then occurring in the control preparation. Hence the reversal of vancomycin inhibition of growth brought about by the addition of a specific peptide to whole cells was also observed in a cell-free preparation synthesizing mucopeptide.

The effect of varying the concentration of added peptide is shown in Table 3. Even at a peptide/vancomycin molar ratio as low as 5.6 there was appreciable alleviation of vancomycin inhibition of mucopeptide synthesis. In cell-free systems, vancomycin is known to induce increased incorporation of radioactive mucopeptide precursors into the lipid intermediate (Higashi *et al.*, 1967) of mucopeptide biosynthesis (Struve *et al.*, 1966; Matsuhashi *et al.*, 1967; Reynolds, 1971*a*). Just as addition of peptide progressively reversed the vancomycin inhibition of mucopeptide synthesis, so it progressively lessened the vancomycin-induced increase in the formation of radioactive lipid intermediate (Table 3). Thus peptide clearly reverses both of the observed effects of vancomycin on the biosynthesis of mucopeptide.

Cell-free preparations able to synthesize mucopeptide were also obtained from *B. megaterium* by blending a thick suspension (50mg dry wt./ml) with styrene–divinylbenzene co-polymer beads, 50–100 mesh, in a microhomogenizer (Reynolds, 1971*a*). After whole cells and debris sedimentable at 4250*g* for 5min had been discarded, three particulate preparations were made, namely fractions sedimenting at 6500*g* for 5min, 12500*g* for 10min and 38000*g* for 20min. The amount of cell wall present in such preparations was measured by determining the amount of radioactivity present in each fraction

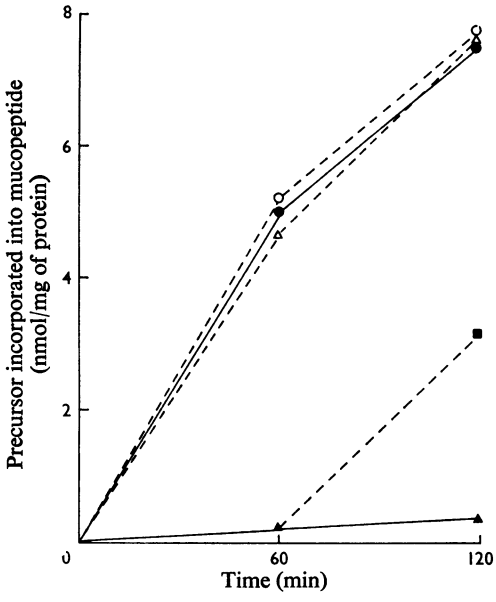

Fig. 5. *Effect of specific peptide on the inhibition by vancomycin of mucopeptide synthesis by a protoplast membrane preparation*

Membranes from 'reconditioned' protoplasts of *B. megaterium* were incubated at 23°C with radioactively labelled precursor as described in the Methods section and the radioactivity incorporated into material remaining at the origin of chromatograms was measured. Vancomycin (final concn. 100μg/ml, 0.056mM) was added to the incubation mixture at 0°C before incubation, and peptide (final concentration 3.13mM) was added either 5min later, also at 0°C, or at 60min after the commencement of incubation. Sample with no additions (●); with vancomycin alone (▲); with peptide alone (○); with vancomycin and peptide added before incubation (△); with vancomycin throughout and peptide added at 60min (■). The peptide/vancomycin molar ratio was 56.

after labelling bacteria with diamino[^{3}H]pimelic acid in the presence of a high concentration of lysine (Reynolds, 1971*a*). Under these conditions 99% of the radioactivity incorporated remains as diaminopimelic acid in the cell wall and cell-wall precursors. The first particulate preparation consisted mainly of cell-wall material (45–50% of the original cell wall) and a little membrane, the second had about half of each, and the third was almost all membrane (less than 1% of the original cell wall). All these preparations synthesized mucopeptide linearly for 80min, the wall-free membranes being the most efficient (Fig. 6). As with the membrane preparation from protoplasts, the inhibitory effect of vancomycin was largely reversed by the addition of specific peptide, whether the peptide was added in the cold 5min after the vancomycin or 40min after the commencement of incubation. The particular enzyme preparation used affected neither the inhibition nor the reversal. When, however, vancomycin was included in the breakage medium, a different result was obtained. After cells had been broken with or without vancomycin (10μg/mg dry wt. of cells; 500μg/ml) and the whole cells had been discarded as described above, two fractions were prepared, sedimenting at 12000*g* for 10min (about 75% wall and 25% membrane) (P1), and 38000*g* for 20min (almost all membrane) (P2). As before, both fractions synthesized mucopeptide, but, of the preparations made from the cells broken in the presence of vancomycin, preparation P1 showed only 24% of the activity of the corresponding control, whereas preparation P2 showed 64% (Fig. 7). Thus the persistence of inhibition derived from pretreatment with vancomycin may be related to the presence of cell-wall material in the preparation, since membranes prepared from protoplasts that had been 'reconditioned' in the presence of vancomycin synthesized mucopeptide without any inhibition, so long as the antibiotic was not added until after 'reconditioning' had commenced (Reynolds, 1971*b*) Fig. 7 also shows that the inhibition of mucopeptide synthesis by particulate preparations from cells pretreated with vancomycin was completely reversible by specific peptide.

The distribution of vancomycin in broken-cell fractions pretreated with antibiotic was followed by the use of [^{125}I]iodovancomycin (Table 4). As expected, most of the antibiotic became attached to those fractions containing cell walls, so that the preparation P1 corresponding to that used for mucopeptide biosynthesis in Fig. 7 contained 36.5% of the total iodovancomycin present, whereas preparation P2, which sedimented at 38000*g* but not at 12000*g*, contained only 0.6% of the antibiotic added. Only a small proportion of the iodovancomycin originally sedimenting with preparation P1 was removed by washing in buffer. Samples of these preparations were incubated under conditions similar to those used for the measurement of mucopeptide synthesis, as in Fig. 7 (Table 5). The total final concentration of iodovancomycin in an appropriate sample of preparation P2 (final volume 25μl) was only 22μg/ml and the corresponding inhibition of mucopeptide synthesis was 30% (Fig. 7). This corresponds fairly closely to the 20% inhibition of mucopeptide synthesis induced by the addition of 20μg of vancomycin/ml to the assay system (Reynolds, 1971*a*). Preparation P1, on the other hand, retained iodovancomycin to a final concentration of 600μg/ml. Incubation in the presence of added mucopeptide precursor, but not otherwise, extracted 31% of the bound antibiotic. Even supposing that some of the residual antibiotic was

Table 3. *Effect of peptide concentration on the reversal of vancomycin effects on mucopeptide synthesis*

Membranes of *B. megaterium* protoplasts were used as described in Fig. 5. Vancomycin (100 μg/ml) and different concentrations of peptide were added before incubation. Radioactivity incorporated from UDP-*N*-acetyl-muramyl-pentapeptide (labelled with diamino[^{3}H]pimelic acid) into mucopeptide and lipid intermediate was measured. Values are percentages of the incorporation observed with a control sample without added vancomycin or peptide.

Additions	Peptide/vancomycin molar ratio	Radioactivity incorporated	
		Mucopeptide	Lipid intermediate
None		100	100
Vancomycin	—	11	163
Vancomycin+peptide	5.6	25	146
	11.2	29	153
	22.5	43	134
	33.7	60	128
	45	68	108
	56	74	104

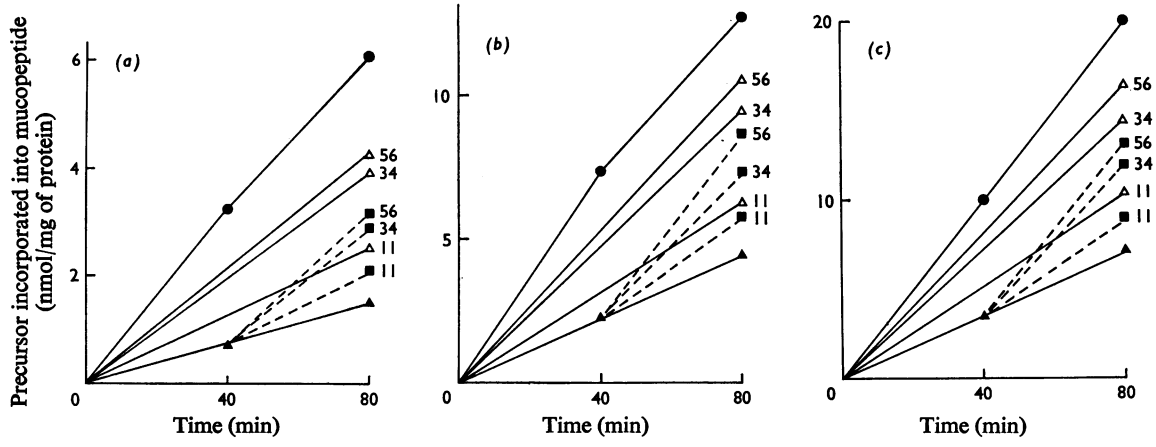

Fig. 6. *Effect of specific peptide on the inhibition by vancomycin of mucopeptide synthesis by broken-cell preparations*

B. megaterium growing exponentially was blended with co-polymer beads. Material sedimenting at 4250*g* for 5 min was discarded and successive fractions sedimenting at (*a*) 6500*g* for 5 min (preparation P1), (*b*) 12500*g* for 10 min (preparation P2) and (*c*) 38000*g* for 20 min (preparation P3) were washed twice in 0.05 M-tris–HCl buffer (pH 7.8)–10 mM-Mg^{2+} and incubated at 23°C with radioactively labelled precursor as described in the Methods section. The radioactivity incorporated into material remaining at the origin of chromatograms was measured. Vancomycin (final concn. 100 μg/ml) was added to the incubation mixture at 0°C before incubation and peptide was added either before, or 40 min after the commencement of, incubation. Samples with no additions (●); with vancomycin alone (▲); with vancomycin and peptide added before incubation (△); with vancomycin throughout and peptide added at 40 min (■). The numbers beside the curves indicate the peptide/vancomycin molar ratio. At a molar ratio of 56 the peptide concentration was 3.13 mM. Note that different scales have been used on the ordinates of the three graphs.

bound to insensitive sites, as suggested above, the final concentration of iodovancomycin in the supernatant solution (184 μg/ml) was high enough to produce considerable inhibition of mucopeptide synthesis (76% in Fig. 7). Reynolds (1971*a*) found that addition of 100 μg of vancomycin/ml to the assay system produced 70% inhibition. The behaviour of broken-cell preparations pretreated with antibiotic in biosynthesis of mucopeptide is thus consistent with the observed retention of iodovancomycin.

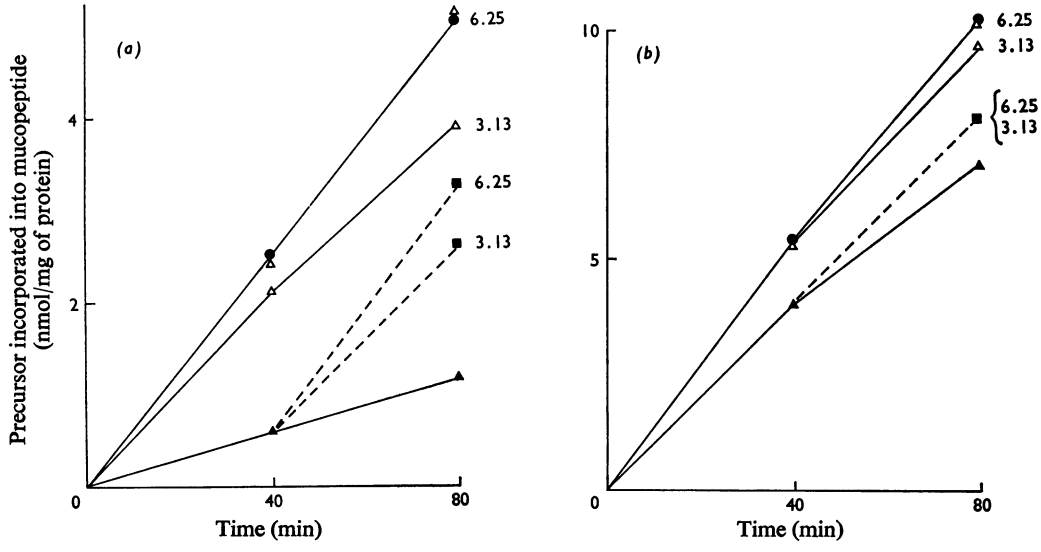

Fig. 7. *Effect of pretreatment with vancomycin on the mucopeptide synthetic activity of broken-cell preparations*

B. megaterium growing exponentially was blended with co-polymer beads either in the absence or in the presence of vancomycin (10μg/mg cell dry wt.; 500μg/ml). Material sedimenting at 4250*g* for 5min was discarded and successive fractions sedimenting at (*a*) 12000*g* for 10min (preparation P1) and (*b*) 38000*g* for 20min (P2) were washed twice in 0.05M-tris–HCl buffer (pH7.8)–10mM-Mg^{2+}, incubated, and radioactivity was measured as described in Fig. 6. The protein concentration in the assay was 3.5mg/ml (P1) and 7.0mg/ml (P2). To the preparations from cells pretreated with vancomycin, specific peptide was added either before incubation or at 40min. Preparations not pretreated with vancomycin (●); preparations pretreated with vancomycin without further addition (▲); with peptide added before incubation (△); with peptide added at 40min (■). The numbers beside the curves indicate the final concentration of peptide (mM). Note that different scales have been used on the ordinates of the two graphs.

Table 4. *Distribution of iodovancomycin in fractions of broken cells prepared from B. megaterium pretreated with antibiotic*

Cells from a culture growing exponentially were blended with co-polymer beads, [^{125}I]iodovancomycin was added (broken cells. 400mg dry wt. in 22.5ml of 0.05M-tris–HCl buffer, pH7.8, 10mM-Mg^{2+}; iodovancomycin, 11.8μg/mg dry wt. of cells, total radioactivity 7.67×10^4 d.p.s.) and the mixture was kept at 0°C for 15min. It was then centrifuged, and successive fractions were collected and their radioactivities were measured. Fractions P1 and P2 were washed in 30ml portions of the same buffer before final measurement of radioactivity.

Fraction	Sedimentation	$10^{-4} \times$ Radioactivity (d.p.s.)	Percentage of total iodovancomycin found in fraction
PO	4250*g*, 5min	2.76	36.6
P1, final	12000*g*, 10min (four times in all)	2.75	36.5
P1, first wash		0.24	
P1, second wash		0.20	7.3
P1, third wash		0.11	
P2, final	38000*g*, 20min (three times in all)	0.048	0.6
P2, first wash		0.075	1.3
P2, second wash		0.026	
Final supernatant solution	Not sedimented at 38000*g*, 20min	1.33	17.5
	Total recovered	7.54	98.4

Table 5. *Effect of incubation on retention of iodovancomycin by broken-cell preparations from B. megaterium*

The fractions obtained by differential centrifuging (Table 4) were resuspended in the same buffer (P1, 1.12ml; P2, 0.53ml) and samples (10μl) were incubated under conditions similar to those used for measurement of incorporation of mucopeptide precursor (Fig. 7), final volume 25μl. The protein concentrations in the assay were 3.7mg/ml (P1) and 6.7mg/ml (P2). After incubation at 23°C for 1.5h the samples were centrifuged at 38000*g* for 20min and the radioactivity of the pellet and the supernatant solution was measured.

Expt. no.	Additions	Fraction	Radioactivity (d.p.s.)	Iodovancomycin (μg)
1	None	P1	246	15.1
2	0.25M-tris–HCl buffer (pH7.8)–20mM-Mg^{2+} (10μl)	P1, supernatant	8.3	0.51
		residue	242	15.0
3	As in Expt. 2+UDP-*N*-acetylglucosamine, final concn. 0.4mM	P1, supernatant	7.5	0.46
		residue	237	14.6
4	As in Expt. 2+UDP-*N*-acetylmuramyl-pentapeptide, final concn. 0.4mM	P1, supernatant	76	4.6
		residue	165	10.1
5	As in Expt. 4+UDP-*N*-acetylglucosamine, final concn. 0.4mM	P1, supernatant	73	4.5
		residue	167	10.3
6	None	P2	9.2	0.56

Discussion

Sinha & Neuhaus (1968) first demonstrated the reversal of the inhibition by vancomycin of mucopeptide synthesis occurring in cell-free system from *S. aureus* and *M. lysodeikticus*. In their experiments the reversing agent was a cell-wall preparation from *M. lysodeikticus*, and they also showed that the addition of cell walls would prevent the accumulation of lipid intermediate brought about by the presence of vancomycin (translocase:transfer assay). They concluded that 'cell walls appear to have a higher affinity for vancomycin than does the peptidoglycan synthetase-acceptor system associated with the membranes'. The present experiments with cell-free preparations from *B. megaterium* show that a peptide, which mimics the non-cross-linked termini present in cell walls by ending in D-Ala-D-Ala, will also counteract vancomycin both in its inhibition of mucopeptide synthesis and in its enhancement of lipid-intermediate accumulation. It is known from previous work (Perkins, 1969; Nieto & Perkins, 1971*b*) that peptides of this type have a high affinity for vancomycin, and it is reasonable to suppose that the added peptide competes with some component of the enzyme preparation for the available antibiotic, thus reversing inhibition. The fact that the extent of reversal was proportional to the concentration of peptide (Fig. 6) supports this view. Although various amounts of cell wall were present in the preparations used, the extent of reversal at a given peptide concentration was the same for all of them, which implies that the affinity for vancomycin of the sensitive site was unchanged. Since at present the concentration of sensitive sites in the incubation mixture is not known, it is not possible to calculate the dissociation constant of their complex with vancomycin, even though the constant for the peptide used has been calculated (Nieto & Perkins, 1971*b*).

Experiments in which vancomycin was present with broken cells but not in the final incubation showed that the observed inhibition of mucopeptide synthesis was related to the amount of cell-wall material in the preparation, which in turn controlled the amount of vancomycin retained. Even this inhibition caused by retained antibiotic was reversible by addition of specific peptide to the final incubation mixture. The mucopeptide precursor present in the assay system itself (10nmol) caused elution of some of the adsorbed antibiotic (2.5nmol) (Table 5) but this extraction was clearly insufficient to relieve the inhibition (Fig. 6). Indeed, the final concentration of vancomycin in the supernatant solution was more than sufficient to cause inhibition of mucopeptide synthesis. Prior adsorption of vancomycin also inhibited the cell-free biosynthetic system obtained from *M. lysodeikticus* (Bordet & Perkins, 1970).

The reversal of vancomycin effects brought about by the addition of peptide to cell-free systems was also observed in living bacteria. When peptide was added to a culture of *B. megaterium* at various intervals after the addition of vancomycin, the growth inhibition caused by the antibiotic was reversed. The time required for resumption of rapid growth increased with the lapse of time before the peptide was added, which implies that the instantaneous inhibition of mucopeptide synthesis caused by vancomycin (Jordan, 1965) was just as rapidly reversed by adding vancomycin-binding peptide but that some more obstinate inhibitory effect arose on prolonged contact with the antibiotic. Whatever this effect was, incubation with peptide eventually reversed it. Since

recovering cells and cells protected from inhibition by the presence of peptide nevertheless took up iodovancomycin, the implication is that bacteria contain some sites for vancomycin binding that compete for the antibiotic with the specific peptide in solution but are not concerned in its inhibitory action. On the other hand, the peptide is clearly able to free the sensitive sites within the cell from bound vancomycin, thus reversing the inhibition of growth.

These results contrast with those of Best *et al.* (1970), who found that the inhibition of growth of *Bacillus subtilis* W23 caused by even a minimum concentration of vancomycin was not reversed by 8mol.prop. of UDP-*N*-acetylmuramyl-pentapeptide. The molecular weight of vancomycin was assumed to be 3200, compared with the value of 1800 used here (Nieto & Perkins, 1971*a*). The fact, therefore, that Best *et al.* (1970) used only 4.5mol.prop. of peptide compared with the 38mol.prop. used in our experiments may account for the apparent discrepancy in results. An alternative explanation would be that the highly acidic nucleotide precursor did not reach the relevant region of the cells so easily as the weakly acidic peptide diacetyldiaminobutyryl-D-alanyl-D-alanine.

Experiments with doses of radioactive iodovancomycin near the minimum inhibitory concentration showed that after the onset of inhibition some further uptake of antibiotic occurred (Fig. 3). Before rapid growth recommenced the excess of antibiotic was released from the cells, but the retained concentration was about the same as when growth inhibition first occurred. One must conclude that sensitive sites in the bacteria had been released from vancomycin inhibition and that the antibiotic retained must have been bound at some insensitive region of the cells. The fact that inhibited cells incubated in buffer released much of their antibiotic along with some material absorbing at 260nm and containing bound *N*-acetylhexosamine, much of which would be UDP-*N*-acetylmuramyl-pentapeptide (Reynolds, 1971*a*), suggests that under those conditions vancomycin may have been flushed out by leaking precursor nucleotide. This process may also occur in bacteria in growth medium. Reynolds (1964) showed that when *S. aureus* cells that accumulated mucopeptide precursor in the presence of vancomycin were resuspended in medium and incubated in the absence of antibiotic, the precursor disappeared from the cytoplasm and part of it was incorporated into the cell wall. Such a process in the presence of limiting concentrations of vancomycin, as in our experiments with *B. megaterium*, could perhaps have the effect of removing the antibiotic from sensitive sites. New acceptor sites may also have been created by the limited amount of mucopeptide synthesis that persists in the presence of vancomycin.

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