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Specificity of Combination between Mucopeptide Precursors and Vancomycin or Ristocetin

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Vancomycin and ristocetin formed complexes on being mixed with mucopeptide precursors from various bacteria, as shown by chromatography, electrophoresis and differential ultraviolet spectra. Equimolar proportions of antibiotic and peptide were present. The specificity of the reaction was studied and the smallest molecule found to react was acetyl-D-alanyl-D-alanine. This *C*-terminal dipeptide sequence must be present for complex-formation; change of configuration or esterification prevented it. Modified vancomycins that retained antibiotic activity also combined with appropriate peptides. The dissociation constants of the more stable complexes were estimated from the differential-absorption results. The relationship of complex-formation to antibiotic action is discussed. Penicillin, supposed to be an analogue of acyl-D-alanyl-D-alanine, also modified the spectrum of vancomycin; so, too, did sodium benzylpenicilloate.

The antibiotics vancomycin and ristocetin, obtained respectively from cultures of *Streptomyces orientalis* and *Nocardia lurida*, are active against Gram-positive rather than Gram-negative bacteria. Both have a molecular weight in the range 3000–5000 and are amphoteric substances containing amino and phenolic groups and sugar residues (for review see Lightbown, 1964). In vancomycin the sugar is glucose, and a partially degraded product lacking glucose is still antibiotically active (Marshall, 1965). Ristocetins A and B contain glucose, mannose, arabinose and rhamnose, but again acid hydrolysis liberates the sugars and leaves a residue that is still an antibiotic, in this case having enhanced activity (Philip, Schenck, Hargie, Holper & Grundy, 1960). The detailed chemistry of these antibiotics is not known, although vancomycin is reported to contain phenols, chlorophenols, aspartic acid and *N*-methyl-leucine (Marshall, 1965).

Antibiotics that inhibit the biosynthesis of the mucopeptide of bacterial cell walls often cause the accumulation of UDP-*N*-acetylmuramyl-peptide precursors, and this is true of ristocetin (Wallas & Strominger, 1963) and vancomycin (Reynolds, 1961, 1966; Jordan, 1961). Further, the biosynthesis of mucopeptide in cell-free systems from *Staphylococcus aureus* and *Micrococcus lysodeikticus* was 50% inhibited by about the same concentrations of vancomycin or ristocetin as were required to inhibit growth by 50% (Anderson, Matsuhashi, Haskin & Strominger, 1965, 1967). There is also some evidence that incorporation of labelled precursors as far as the lipid-intermediate stage of mucopeptide biosynthesis is actually enhanced in the presence of these antibiotics (Matsuhashi, Dietrich & Strominger, 1967; Chatterjee, Ward & Perkins, 1967).

Thus the connexion between mucopeptide biosynthesis and the action of vancomycin and ristocetin is clear, but the precise mode of action is not known. An additional observation was made by Chatterjee & Perkins (1966*a*), who found that Gram-positive bacteria accumulating nucleotide-*N*-acetylmuramyl-peptide precursors in the presence of vancomycin or ristocetin also contained the same precursors linked to the antibiotics. At that time the results appeared to show that a cell-free extract was required to unite vancomycin to the nucleotide precursor, but it is now shown that the two molecules combine on mixing, as briefly described previously (Perkins, 1968). The specificity of the reaction is also further examined.

METHODS

Antibiotics. Vancomycin hydrochloride (Vancocin HCl) was kindly given by Eli Lilly and Co. Ltd., Basingstoke, Hants., and ristocetin A (Spontin) by Abbott Laboratories, N. Chicago, Ill., U.S.A. Sodium benzylpenicillin was obtained from Glaxo Laboratories Ltd., Greenford, Middx., and D-cycloserine from Sigma Chemical Co., St Louis, Mo., U.S.A. 6-Aminopenicillanic acid was a gift from Mr F. R. Batchelor, Beecham Research Laboratories, Brockham Park, Betchworth, Surrey.

Mucopeptide precursors and related compounds. Alanine dipeptides were commercial samples and alanine tri- and tetra-peptides of known configuration (Schechter & Berger, 1966) were kindly given by Dr I. Schechter. L-Isoglutaminyl-L-lysyl-D-alanyl-D-alanine and L-alanyl-D-isoglutaminyl-L-lysyl-D-alanine were gifts from Mr P. Lefrancier (Munoz *et al.* 1966). UDP-*N*-acetylmuramyl-L-alanyl-D-glutamyl-*meso*-diaminopimelyl-D-alanyl-D-alanine and UDP-*N*-acetylmuramyl-L-alanyl-D-glutamyl-*meso*-diaminopimelic acid from *Bacillus licheniformis* were given by Dr A. J. Garrett, and 4-*O*-β-*N*-acetylglucosaminyl-*N*-acetylmuramyl-L-alanyl-D-glutamyl-*meso*-diaminopimelyl-D-alanine was prepared by Dr H. Pelzer and given by Dr R. C. Hughes. UDP-*N*-acetylmuramyl-L-glycyl-D-glutamyl-L-diaminobutyryl-D-alanyl-D-alanine was obtained from a culture of *Corynebacterium tritici* (N.C.P.P. 471) as described by Chatterjee & Perkins (1966*a*). UDP-*N*-acetylmuramyl-tripeptides (Table 1) were prepared by treating exponential-phase cells with D-cycloserine (80μg./ml.) and then following the procedure of Chatterjee & Perkins (1966*a*).

Paper chromatography and electrophoresis. Whatman no. 3 paper was used, and if required for preparative purposes it was first exhaustively washed by irrigation with 1M-ammonium acetate followed by water. Nucleotides were separated by chromatography in solvent 1 (ethanol–1M-ammonium acetate, 5:2, v/v) or solvent 2 (isobutyric acid–aq. 0·5M-NH_3, 5:3, v/v). The following buffers were used for electrophoresis: buffer A, collidine–acetate, pH 7 (Newton & Abraham, 1954); buffer B, pyridine–acetate, pH 4·6 (acetic acid–pyridine–water, 1:1:200, by vol.); buffer C, as buffer B, but containing 1M-NaCl; buffer D, 0·01M-calcium acetate brought to pH 4·6 with acetic acid; buffer E, 0·25M-formic acid.

Compounds were observed on chromatograms by u.v. absorption (nucleotides) or 0·1% ninhydrin in acetone (peptides). Vancomycin derivatives could be seen as faint u.v.-absorbent regions, but they also stained orange–yellow with the acid–diazosulphanilic acid reagent of Ames & Mitchell (1952).

Degradation of mucopeptide precursor. UDP-*N*-acetylmuramylglycyl-D-glutamylhomoseryl-D-alanyl-D-alanine (Chatterjee & Perkins, 1966*a*,*b*; Perkins, 1967) (0·7μmole) was heated in 0·05N-HCl (0·5ml.) at 100° for 5min. to remove the UDP. After the solution had been dried, the residue was transferred to washed paper and subjected to electrophoresis in buffer B at 10v/cm. for 2·5hr. The released UDP was the main u.v.-absorbing spot (there was a trace of UMP as well) and the remainder of the molecule was detected by the spray reagent of Partridge (1948) for acetamido sugars. *N*-Acetylmuramylglycyl-D-glutamylhomoseryl-D-alanyl-D-alanine was eluted with water.

UDP-*N*-acetylmuramyl-L-alanyl-D-glutamyl-L-lysyl-D-alanyl-D-alanine was obtained from a culture of *S. aureus* as described by Chatterjee & Perkins (1966*a*). A sample (1μmole) was heated with 0·05N-HCl (0·5ml.) at 100° for 5min. to remove UDP, and the resulting solution was made alkaline to phenolphthalein with 0·5M-Na_2CO_3 and then a further 2 drops were added. It was then heated at 100° for 4min. to remove as chromogen the *N*-acetylglucosaminyl part of the muramic acid residue (Perkins, 1967; Ghuysen, Bricas, Leyh-Bouille, Lache & Shockman, 1967; Tipper, 1968). The product was transferred to a column (2ml.) of Zeo-Karb 225 (H^+ form) and eluted with aq. 2N-NH_3. The material was subjected to paper electrophoresis in buffer B, and the ninhydrin-positive band at the origin was eluted. A sample was analysed for amino acids and found to be consistent with the composition lactyl-L-alanyl-D-glutamyl-L-lysyl-D-alanyl-D-alanine.

Acylation of vancomycin. Vancomycin hydrochloride (9mg.) in a stoppered tube was treated with 0·2ml. of formic acid (98%) cooled in ice. Acetic anhydride (70μl.) was added slowly and then the mixture was allowed to come to room temperature and left for 4hr. The sample was dried *in vacuo*, brought to pH 10 with NH_3 and incubated at 37° for 1hr. to remove any *O*-formyl groups. The formylated product was freed from unchanged vancomycin by paper electrophoresis.

Vancomycin hydrochloride (15mg.) was dissolved in water (140μl.) and cooled in ice. Saturated $NaHCO_3$ solution (60μl.) was added, followed by the slow addition of acetic anhydride (60μl.). After 1hr. in the ice bath the mixture was kept at room temperature for 0·5hr. and then dried in a vacuum. Any *O*-acetyl groups were removed by NH_3 treatment as above.

The formylated and acetylated vancomycin samples were separated by paper electrophoresis in buffer B. Unchanged vancomycin moved as a cation (compared with glucose used as a neutral marker), the acetylated product contained mainly a well-defined neutral band, and the formylated sample had one neutral and one anionic band. The samples were eluted, dried and dissolved in water, and solution was completed by the addition of a little aq. NH_3. The concentration was determined by measuring E_{282} and assuming that the molar extinction coefficient was the same as that for unchanged vancomycin hydrochloride (ϵ 6100 assuming mol.wt. 1600; see the Results section).

Antibiotic potency of acylated vancomycin. Plates were prepared from broth–agar containing 1% (w/v) of glucose, inoculated with a suspension of *Corynebacterium poinsettiae* (N.C.P.P. 177). Wells 5mm. in diameter were bored in the agar, and antibiotic solutions of known concentration were introduced. The plates were incubated at 26° and after growth had occurred the diameter of the inhibition zones was measured.

Acetylation and methylation of peptides. Alanine peptides were acetylated by acetic anhydride in the presence of $NaHCO_3$. To avoid racemization the temperature was kept low and a large excess of acetic anhydride was not used. For example, to D-alanyl-D-alanine (66μmoles) was added ice-cold 0·3M-acetic anhydride–0·4M-$NaHCO_3$ (0·39ml.). The mixture was kept at 0° for 3hr., the pH then being 7, and then boiled for 3min. to decompose the excess of acetic anhydride. The material was transferred to a washed paper and subjected to electrophoresis in buffer A at 10v/cm. for 80min. Ninhydrin revealed a small neutral band of unchanged dipeptide, and the main product was an anionic band stained by the method of Rydon & Smith (1952). The acetyl-D-alanyl-D-alanine was eluted and concentrated. A sample was hydrolysed in 6N-HCl at 105° for 16hr., and the alanine content was determined after electrophoresis in buffer E (Perkins, 1965).

Peptides and acetylated peptides were esterified by diazomethane. Thus to acetyl-D-alanyl-D-alanine (3·6 μmoles) in 0·1ml. of water was added 0·8ml. of methanol followed by an ethereal solution of diazomethane until effervescence ceased and a yellow colour persisted. Excess of

reagent was destroyed with acetic acid and the product was dried in a stream of air. Electrophoresis of a sample in buffer A showed that the acidic acyl-peptide had been completely converted into a neutral product detected by the method of Rydon & Smith (1952). The esterified product was further identified by reconversion into acetyl-D-alanyl-D-alanine by incubation at pH 10 and 50° for 3hr.

Dinitrophenylation of vancomycin–nucleotide compound. The compound was dissolved in water and treated in the dark at room temperature with ethanolic 1-fluoro-2,4-dinitrobenzene (0·5%, v/v; 2vol.) and 1M-$NaHCO_3$ (0·025vol.). After 3hr. the ethanol was removed under vacuum. The yellow precipitate of dinitrophenylated vancomycin was removed by centrifuging and the supernatant was then shaken with chloroform–3-methylbutan-1-ol (4:1, v/v). The clear aqueous layer was removed, applied to a column of Sephadex G-25 and eluted with water. The first peak of u.v.-absorbing material contained nucleotide–pentapeptide free of vancomycin, shown by the absence of aspartic acid or other vancomycin breakdown products in hydrolysates. The recovery of N-acetylhexosamine in this peak, measured by the method of Reissig, Strominger & Leloir (1955), was about 60%.

Differential u.v. absorption of antibiotics and peptides. The combination of peptides with vancomycin or ristocetin was measured by observation of changes in the u.v.-absorption spectrum. The concentration of vancomycin hydrochloride found convenient for use in 1cm. cells was 0·272mg./ml. (taken as 0·17mM, see the Results section). The instrument used was a Unicam SP.700 (Unicam Instruments Ltd., Cambridge). In the reference beam were placed one cell containing antibiotic solution (2·5ml.) and one containing water (2·5ml.). In the sample beam were one water cell and another with 2·5ml. of antibiotic solution. Samples of peptide solution (usually 0·02M) were added by Carlsberg pipette to the water cell in the reference beam and to the antibiotic solution in the sample beam. The same volume of water was added to the antibiotic cell in the reference beam. The solutions were mixed and the spectra recorded over the range 260–320nm. The differential absorption where it was greatest (close to 283nm.) was measured on the recorder charts and plotted against the concentration of added peptide. All experiments were performed at room temperature.

Monosodium benzylpenicilloate. This was synthesized as follows. To 375mg. of sodium benzylpenicillin was added 25·3ml. of ice-cold 0·05N-NaOH. The solution was allowed to warm to room temperature and left for 3hr. It was then acidified to pH 5 with HCl and freeze-dried. On addition of 2ml. of water to the dry sample, crystals formed almost immediately, and these were left at 2° overnight. They were filtered on a sintered-glass funnel and washed repeatedly with aq. 90% (v/v) acetone. The fine white needles were dried over P_2O_5. They had m.p. 152·5–153·5° (uncorrected) and $[\alpha]_D^{23}$ +132° (0·47% in 0·2M-phosphate buffer, pH 8). Mozingo & Folkers (1949) quoted m.p. 155–156·5° and $[\alpha]_D^{23}$ +129°.

Preparation of N-*acetylmuramyl-L-alanyl-D-glutamyl-*meso-*diaminopimelyl-D-alanine.* A sample of the 4-*O*-β-*N*-acetylglucosaminyl - *N* - acetylmuramyl - tetrapeptide isolated from the cell walls of *Escherichia coli* by Dr H. Pelzer (Weidel & Pelzer, 1964) was incubated with snail juice (Industrie Biologique Française S.A., Gennevilliers, Seine, France) in 0·1M-sodium acetate buffer, pH 4·6, at 35° for 20hr. The resulting solution was applied to washed paper and chromatographed in solvent 2. All the starting material, which migrated 14·5cm. in this solvent, had disappeared, and a new band at 17·1cm. had appeared that reacted with both ninhydrin and the reagent for acetamido sugars (Partridge, 1948). A band of *N*-acetylglucosamine was also visible at 27·8cm. The desired product was eluted and shown to contain, for each mole of *N*-acetylmuramic acid, 2 moles of alanine and 1 mole each of glutamic acid and diaminopimelic acid. *N*-Acetylhexosamine was determined by the method of Reissig, Strominger & Leloir (1955) and amino acids with a ninhydrin reagent after paper chromatography in butan-1-ol–pyridine–water (6:4:3, by vol.) (Hughes, 1968).

RESULTS

Combination of mucopeptide precursors with vancomycin detected chromatographically. Previous work (Chatterjee & Perkins, 1966*a*) had shown that, after incubation with vancomycin, cells of *C. poinsettiae* contained a compound of that antibiotic and the mucopeptide precursor UDP-*N*-acetylmuramylglycyl-D-glutamylhomoseryl-D-alanyl-D-alanine. Therefore a small quantity of the precursor nucleotide, usually 0·1μmole in about 10μl., was mixed with an equimolar quantity of vancomycin hydrochloride solution, both at neutral pH, the molecular weight of vancomycin hydrochloride for this purpose being taken as 1600 (see below). This is the weight of hydrochloride that contains one residue each of aspartic acid, *N*-methyl-leucine and glucose; the true molecular weight may be twice this value (Johnson, 1962; Marshall, 1965). On mixing, a white precipitate appeared momentarily and rapidly disappeared, leaving a clear solution, pH 7. The sample was applied to a chromatogram and developed in solvent 1 or 2 overnight. In a typical run in solvent 1 vancomycin appeared as a long spot from 12 to 28cm., precursor nucleotide moved 9·7cm. and the compound between them formed in the mixed sample gave only a spot at the origin, no free vancomycin or nucleotide being present.

The mucopeptide precursors from various bacteria were examined by this procedure, together with some incomplete precursors lacking the terminal alanine dipeptide that had been accumulated in the presence of D-cycloserine (Strominger, Threnn & Scott, 1959). The formation of vancomycin–nucleotide compounds is shown in Table 1. It was clear that the complete precursor nucleotide would combine with vancomycin, regardless of variation in the amino acids present at the *N*-terminal end of the pentapeptide or in the middle positions. In the absence of the *C*-terminal D-alanyl-D-alanine sequence, no combination occurred.

The necessity for the presence of the UDP end

Table 1. *Formation of compounds between vancomycin and uridine nucleotides*

Combination was indicated by formation of material moving slowly on chromatography in solvent 1. Abbreviations: Mur*N*Ac, *N*-acetylmuramyl; Hsr, homoserine; Dap, 2,6-diaminopimelic acid; Dab, 2,4-diaminobutyric acid.

Organism	Nucleotide	Compound formed with vancomycin
C. poinsettiae	UDP-Mur*N*Ac-Gly-D-Glu-Hsr-D-Ala-D-Ala*	Yes
	UDP-Mur*N*Ac-Gly-D-Glu-Hsr	No
S. aureus	UDP-Mur*N*Ac-L-Ala-D-Glu-L-Lys-D-Ala-D-Ala	Yes
	UDP-Mur*N*Ac-L-Ala-D-Glu-L-Lys	No
B. licheniformis	UDP-Mur*N*Ac-L-Ala-D-Glu-*meso*-Dap-D-Ala-D-Ala	Yes
	UDP-Mur*N*Ac-L-Ala-D-Glu-*meso*-Dap	No
C. tritici	UDP-Mur*N*Ac-Gly-Glu-Dab-D-Ala-D-Ala	Yes
Control compounds	UDP-*N*-acetylglucosamine	No
	UDP-glucose	No

* Combination between this nucleotide and ristocetin A was demonstrated in the same way.

of the molecule was then studied. *N*-Acetylmuramylglycyl-D-glutamylhomoseryl-D-alanyl-D-alanine and the related compound from *S. aureus* were prepared by brief acid hydrolysis of the complete nucleotide, and samples were mixed with vancomycin and applied to a chromatogram. Once more the free vancomycin and the fast-running peptides disappeared and a new slower elongated spot appeared that was u.v.-absorbent (because of its vancomycin content) and that gave the reaction for acetamido sugar. The presence of vancomycin in the compound spot was confirmed by the use of the diazosulphanilic acid reagent.

Precursor nucleotide from *S. aureus* from which the UDP had been removed was further degraded in alkali to yield lactyl-L-alanyl-D-glutamyl-L-lysyl-D-alanyl-D-alanine. Mixing with a three-molar excess of vancomycin and chromatography gave a compound; the following were the R_F values in solvent 2: lactyl-peptide, 0·68; vancomycin, 0·62; compound, 0·43. Free peptide had completely disappeared from the mixture. From these results it was clear that the specificity for combination with vancomycin lay towards the *C*-terminal end of the precursor molecule, but it was not known whether both terminal alanine residues were required, or how much of the rest of the molecule was needed.

A different portion of one of the precursors was obtained by enzymic degradation of a product from a lysozyme digest of the mucopeptide from *E. coli*. The resulting *N*-acetylmuramyl-L-alanyl-D-glutamyl-*meso*-diaminopimelyl-D-alanine was mixed with vancomycin and chromatographed in solvent 2. No compound formation at all was observed. Since the corresponding complete nucleotide combined with vancomycin (Table 1), and removal of UDP did not prevent combination with the nucleotide from *S. aureus*, it seemed likely that both the *C*-terminal D-alanine residues were required for combination.

As the nucleotide–pentapeptide from *C. poinsettiae* combined with vancomycin (Table 1), it was known that a free amino group on the molecule was not necessary for combination. In fact, it was found that dinitrophenylation of the nucleotide–pentapeptides from *S. aureus* and *B. licheniformis* also did not prevent combination, indicating that introduction of the bulky DNP group on the central amino acid residue did not interfere with the specificity for vancomycin.

Stability of vancomycin–nucleotide compounds. Attempts were made by various methods to dissociate the compound between vancomycin and UDP-*N*-acetylmuramylglycyl-D-glutamyl-homoseryl-D-alanyl-D-alanine. The possibility of a simple ionic bond seemed unlikely because of the observed degree of specificity for the peptide structure. In addition, the compound was subjected to paper electrophoresis in a solution containing 1M-sodium chloride (buffer C) for 12hr. at 0·9v/cm. Free nucleotide moved further towards the anode and free vancomycin further towards the cathode; there was no sign of dissociation of the compound. This result further discounted the idea of a simple polar link.

Best & Durham (1965) showed that whole cells or isolated cell walls of *Bacillus subtilis* adsorbed vancomycin and that this adsorption was reversed by Mg^{2+} or Ca^{2+} (1·6mM). To see if Ca^{2+} caused the vancomycin–nucleotide compound to dissociate, a sample was treated by paper electrophoresis in buffer D containing 0·01M-Ca^{2+}. No decomposition to the constituent molecules occurred. Similarly, chromatography in solvent 1 modified to include 0·085M-Mg^{2+} did not dissociate the vancomycin–

Table 2. *Antibiotic action of acylated vancomycin*

The values give the diameter (cm.) of the observed zones of growth inhibition of *C. poinsettiae* surrounding wells (0·5cm. diam.) in which the solutions were applied.

Substance	Concn. (μg./ml.) ...	Diameter (cm.)			
		12·5	25	50	100
Vancomycin hydrochloride		1·3	2·1	2·0	2·6
Acetylated vancomycin		0·8	1·0	1·4	1·8
Formylated vancomycin (neutral band)		0·7	0·9	1·2	1·4
Formylated vancomycin (acidic band)		None	None	0·8	0·8

nucleotide compound (Chatterjee & Perkins, 1966*a*).

If the link between the antibiotic and the appropriate peptides were due to hydrogen-bonding, dissociation in the presence of 8M-urea might be expected. Buffer B containing 8M-urea was used for paper electrophoresis of the vancomycin–nucleotide compound at 10v/cm. for 2·5hr. Almost complete dissociation into an anionic spot similar to the marker nucleotide and a cationic spot like vancomycin occurred. Attempts were made to use this dissociation in urea as the basis for a separation of nucleotide and vancomycin on Sephadex G-25 and G-50 columns, but in practice the dissociated species did not separate well enough to allow a suitable fractionation.

A mild procedure that could be used for separation was dinitrophenylation. This only yielded unchanged nucleotide, however, if the molecule happened to contain no free amino group, as in the nucleotide–pentapeptide from *C. poinsettiae* (Table 1). The method depended on the fact that dinitrophenylated vancomycin was no longer able to combine with peptides and was also rather insoluble. If the compound derived from *S. aureus* were used, for instance, then the dissociated product could be identified as UDP-*N*-acetylmuramyl-L-alanyl-D-glutamyl-ε-DNP-L-lysyl-D-alanyl-D-alanine.

Modified vancomycins. As already indicated, dinitrophenylation of vancomycin precluded combination with peptides. To test whether free amino groups of vancomycin were essential for combination, the antibiotic was formylated or acetylated. The less basic products, separated on electrophoresis but not further characterized, were mixed with nucleotide–pentapeptide from *C. poinsettiae* and chromatographed in solvent 1. The acetylated product and the two different formylated products all gave slow spots, indicative of compound formation, with concomitant disappearance of free nucleotide and antibiotic. A parallel experiment in solvent 2 suggested that some dissociation of the compounds might occur in this solvent. If the combination reaction is at all connected with the antibiotic activity of vancomycin, modified forms that combine with nucleotide–peptides should also retain some biological activity. The acylated products were tested at various dilutions against *C. poinsettiae* growing on agar plates. All three inhibited growth, though at higher concentrations than were required for unsubstituted vancomycin (Table 2).

Aglucovancomycin, a form of the antibiotic without glucose but retaining antibacterial activity, was prepared as described by Marshall (1965). Combination with UDP-*N*-acetylmuramylglycyl-D-glutamylhomoseryl-D-alanyl-D-alanine was demonstrated on chromatograms in solvent 1, the slower u.v.-absorbing spot ($R_{\text{free nucleotide}}$ 0·6) also reacting with diazotized sulphanilic acid. Once more a similar experiment in which solvent 2 was used showed very little apparent combination.

Ultraviolet absorption of vancomycin–peptide compounds. The absorption spectrum of vancomycin has a peak at about 280nm., attributable to the presence of phenolic groups, and a very high absorption below 240nm. (Higgins, Harrison, Wild, Bungay & McCormick, 1958) (Fig. 1*a*). Combination between vancomycin and peptides could be demonstrated by examination of the differential absorption spectrum in the ultraviolet, a typical example being given in Fig. 1(*b*). The trough in the differential curve occurred at 282nm., whereas the maximum in the absorption curve of vancomycin was at 280nm.

When a suitable peptide was added to vancomycin solution, the development of the minimum in the differential absorption curve at about 282nm. could be used as a measure of compound-formation. The differential absorption was plotted against the molar proportion of peptide added to the vancomycin. The curves for the precursor nucleotide from *C. poinsettiae* and for the same molecule without UDP are given in Fig. 2. Essentially similar results were obtained for corresponding nucleotides of *S. aureus* (Fig. 3), *B. licheniformis* and *M. lysodeikticus*. In each instance the curve levelled off when 1 molar proportion of nucleotide had been added, suggesting that one unit of vancomycin that contained one residue each of glucose, aspartic acid and *N*-methyl-leucine (taken here as one molecule,

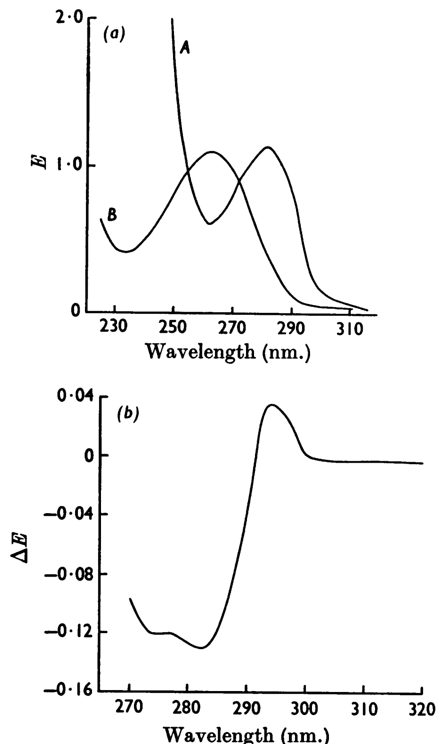

Fig. 1. (*a*) Extinction curves of vancomycin hydrochloride (curve *A*) and UDP-*N*-acetylmuramyl-L-alanyl-D-glutamyl-L-lysyl-D-alanyl-D-alanine (curve *B*), both substances in neutral solution at 0·17mM. (*b*) Differential absorption curve of the compound formed between vancomycin and UDP-*N*-acetylmuramyl-L-alanyl-D-glutamyl-L-lysyl-D-alanyl-D-alanine. The sample cell (1cm.) contained a mixture of vancomycin hydrochloride (0·17mM) and nucleotide (0·16mM). The two cells in the reference beam contained the same solutions separately.

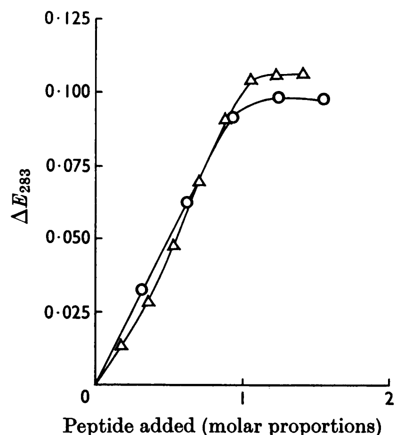

Fig. 2. Differential absorption of vancomycin during addition of peptides from *C. poinsettiae*. The sample cell contained vancomycin (0·17mM) and measured volumes of peptide (0·02M) were added. Cells in the reference beam contained the same solutions separately. The measured differential absorption at the minimum (283nm.) was corrected for the slight dilution. △, UDP-*N*-acetylmuramylglycyl-D-glutamylhomoseryl-D-alanyl-D-alanine; ○, *N*-acetylmuramylglycyl-D-glutamylhomoseryl-D-alanyl-D-alanine.

as mentioned above) could combine with one molecule of nucleotide and no more.

The effect on combination of removing residues from the nucleotide end of the molecule was studied by comparison of the nucleotide from *S. aureus* with the synthetic peptide L-isoglutaminyl-L-lysyl-D-alanyl-D-alanine. The *N*-terminal residue of this peptide differed from the corresponding position in the natural compound in two ways: it had the L- instead of the D-configuration and it was amidated on its α-carboxyl group. Nevertheless the synthetic peptide combined well with vancomycin, although not so well as the natural mucopeptide precursor (Fig. 3). The related tetrapeptide L-alanyl-D-isoglutaminyl-L-lysyl-D-alanine, i.e. the amidated form of the four-amino acid sequence next to the muramic acid of the nucleotide–pentapeptide of *S. aureus* and excluding the *C*-terminal D-alanine residue, was used as a control. It gave no differential spectrum with vancomycin, even at ten times the usual concentration of both vancomycin and peptide (1mm.-light-path cells). UDP-*N*-acetylmuramyl-L-alanyl-D-glutamyl-L-lysine behaved similarly, confirming the lack of combination observed chromatographically.

Combination with alanine peptides. A series of alanine oligopeptides of known configuration was synthesized by Schechter & Berger (1966). These peptides were used here to study the specificity of the reaction with vancomycin. Titrations were performed by observation of full differential spectra, which were essentially the same as those obtained with mucopeptide precursors, or by measurement of differential absorption at 283nm. The curves for certain alanine peptides are given in Fig. 4, together with a list of peptides that did not combine. The results show that, to combine, the peptide must terminate in D-alanyl-D-alanine, although this dipeptide alone was without effect even in solutions of ten times the usual concentration. Evidently some substituent of the amino group of the second D-alanine residue was required to allow combination with vancomycin. When the substituent was another alanine residue, then the L-configuration was more effective than the D-configuration. Of the three tetrapeptides that combined at all, the one most closely resembling

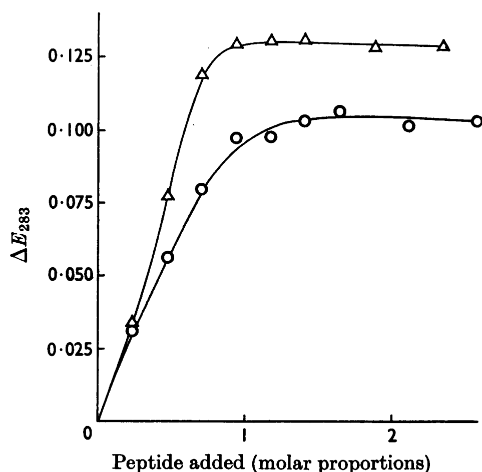

Fig. 3. Differential absorption of vancomycin during addition of *S. aureus* nucleotide and a related tetrapeptide. The sample cell contained vancomycin (0·17mM) and peptide (0·02M) was added. Cells in the reference beam contained the same solutions separately. △, UDP-*N*-acetylmuramyl-L-alanyl-D-glutamyl-L-lysyl-D-alanyl-D-alanine; ○, L-isoglutaminyl-L-lysyl-D-alanyl-D-alanine.

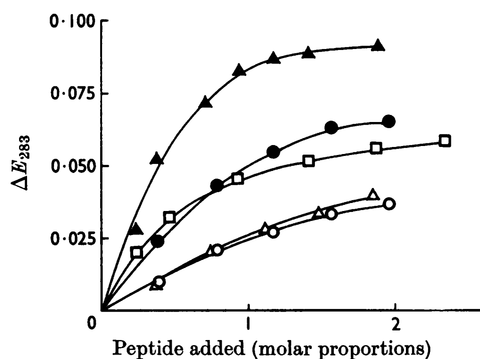

Fig. 4. Differential absorption of vancomycin during addition of alanine peptides. The sample cell contained vancomycin (0·17mM) and peptide (0·02M) was added. Tripeptides: ○, D–D–D; ●, L–D–D. Tetrapeptides: △, D–D–D–D; □, L–D–D–D; ▲, L–L–D–D. The following peptides gave no differential spectrum: dipeptide, D–D; tripeptides, L–L–D, D–D–L and L–D–L; tetrapeptides, L–L–L–D, D–L–L–D and D–D–L–L.

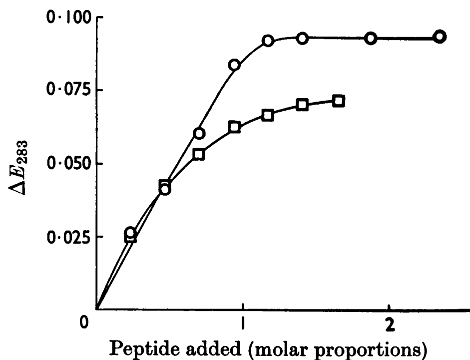

Fig. 5. Differential absorption of vancomycin during addition of acetylated alanine peptides. The sample cell contained vancomycin (0·17mM) and peptide (0·02M) was added. ○, Acetyl-L-alanyl-D-alanyl-D-alanine; □, acetyl-D-alanyl-D-alanine.

the sequence of configurations in the natural mucopeptide precursor, namely L–L–D–D, gave the greatest differential absorption and the sharpest change in slope of the titration curve at about 1 molar proportion. The exact tetrapeptide analogue, D–L–D–D, was not available for comparison.

Since acylation by both L- and D-alanyl residues conferred some combining ability on the inactive D-alanyl-D-alanine, it seemed possible that any substituent blocking the free amino group of the dipeptide might serve. Acetyl-D-alanyl-D-alanine and acetyl-L-alanyl-D-alanyl-D-alanine were synthesized and titrated with vancomycin (Fig. 5). Acetylation of the dipeptide allowed it to react at least as well as acylation by a residue of L-alanine, and similar substitution of the tripeptide (L–D–D) produced a compound that showed a very sharp change in slope of the titration curve at about 1 molar proportion, comparable with the curves for mucopeptide precursors (Figs. 2 and 3). As expected, acetyl-L-alanyl-L-alanine did not react with vancomycin.

The importance for combination of the free carboxyl group was assessed by esterification. Two substances known to combine with vancomycin, acetyl-D-alanyl-D-alanine and acetyl-L-alanyl-L-alanyl-D-alanyl-D-alanine, were each converted into their methyl ester. Neither of these esters combined at all with the antibiotic.

Calculation of dissociation constants. The titration curves shown in Figs. 2–5 can be used for calculation of the dissociation constant for the compound between vancomycin and a peptide, if the assumption is made that the plateau corresponds to 100% combination, when all the vancomycin is in the form of its complex with 1 molar proportion of peptide, and that lower values of the differential absorption are proportional to the concentration of the complex. This assumption was not strictly true, since in some instances the differential absorption with low proportions of added peptide was excessively high. However, if calculations were made when saturation had been more nearly achieved, a series of values was obtained that

Table 3. *Dissociation constants of vancomycin–peptide complexes*

The constants were calculated from the titration curves obtained by using differential absorption, by application of the law of mass action. Thus for *N*-acetylmuramylglycyl-D-glutamylhomoseryl-D-alanyl-D-alanine the plateau in differential absorption was at ΔE_{283} 0·0980. When 1 molar proportion of peptide had been added ΔE_{283} was 0·0955. Hence 2·55% remained unchanged. Since the initial vancomycin concentration was 0·17mM, the final concentration of complex was 0·9745 × 0·17mM, and of free peptide or vancomycin 0·0255 × 0·17mM. The dissociation constant, *K*, was calculated from the expression

$$K = \frac{[\text{Vancomycin}][\text{Peptide}]}{[\text{Complex}]}$$

Substance	*K* (M)
UDP-*N*-acetylmuramylglycyl-D-glutamylhomoseryl-D-alanyl-D-alanine	6×10^{-7}
N-Acetylmuramylglycyl-D-glutamylhomoseryl-D-alanyl-D-alanine	1×10^{-7}
L-Isoglutaminyl-L-lysyl-D-alanyl-D-alanine	2×10^{-6}
L-Alanyl-L-alanyl-D-alanyl-D-alanine	4×10^{-6}
L-Alanyl-D-alanyl-D-alanine	2×10^{-5}
Acetyl-L-alanyl-D-alanyl-D-alanine	8×10^{-7}
Acetyl-D-alanyl-D-alanine	1×10^{-5}

served as some indication of the affinity for vancomycin of the various substrates. Such calculations could not be made with very poor substrates, although sometimes it was possible to obtain suitable curves by using tenfold concentrated vancomycin in a cell of 1mm. light-path. The approximate dissociation constants are given in Table 3. The lowest values were those for the mucopeptide precursors, the analogue approaching nearest being acetyl-L-alanyl-D-alanyl-D-alanine (K 8×10^{-7}M). This was also the structure that fitted most closely to that of the precursors, it being borne in mind that the residue in the fourth position (acetyl) had no free amino group.

Ristocetin. A few experiments with ristocetin showed that its absorption spectrum, too, was modified by the presence of a suitable peptide. Ristocetin B was used at a concentration of 0·3mg./ml. to give E_{280} ~ 1·5. The true molecular weight is not known, since acid titration gave an equivalent weight of 1200–1400, freezing-point depression in aqueous solution suggested a molecular weight of 2500 and the ultracentrifuge gave a value of about 5000 (Philip, Schenck & Hargie, 1957). Later work showed that ristocetin B contained glucose, mannose, rhamnose and arabinose, there being 1] mole of arabinose/unit of molecular weight about 4000 (Philip *et al.* 1960). The differential absorption curve obtained with approximately equimolar proportions of ristocetin B and acetyl-L-alanyl-D-alanyl-D-alanine is shown in Fig. 6. Measurements were made at the differential absorption minimum at 287·5nm. and a titration curve was plotted (Fig. 7). At the concentration used (0·15mM) the total absorption was too great to measure the differential absorption at low wavelengths, but by using more dilute solutions

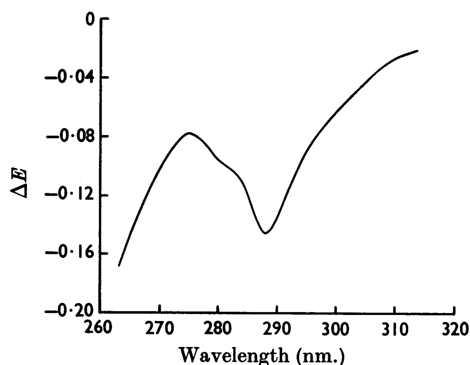

Fig. 6. Differential absorption curve of the compound formed between ristocetin B and acetyl-L-alanyl-D-alanyl-D-alanine. The sample cell contained a mixture of ristocetin B (0·3mg./ml., nominal 0·15mM) and peptide (0·16mM). The two cells in the reference beam contained the same solutions separately.

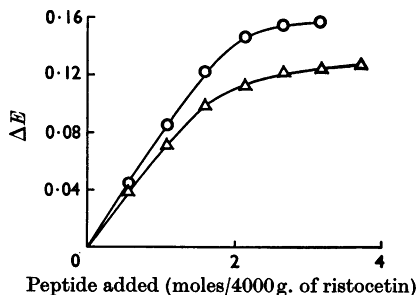

Fig. 7. Titration of ristocetin B with acetyl-L-alanyl D-alanyl-D-alanine. ○, Differential absorption of 0·15mM-ristocetin at 287·5nm.; △, differential absorption of 0·025mM-ristocetin at 243nm.

another minimum was obtained at 243nm. and a titration curve derived (Fig. 7). In a parallel experiment ristocetin was mixed with D-alanyl-D-alanine, but no differential absorption resulted.

Vancomycin and penicillin. The foregoing results show that vancomycin combined with peptides terminating in acyl-D-alanyl-D-alanine, its u.v.-absorption spectrum being modified in consequence. One theory of the mode of action of penicillin that has received much experimental support recently is that, as an analogue of acyl-D-alanyl-D-alanine, it is recognized by enzymes concerned with transpeptidation of mucopeptide chains ending in the same amino acid sequence (Tipper & Strominger, 1965). The penicillin is then supposed to acylate the transfer site of the enzyme, producing an inactive penicilloyl derivative. Similarly, penicillin inhibits a carboxypeptidase of *E. coli* that is specific for removal of the terminal D-alanine residue of non-cross-linked mucopeptide or its precursors (Izaki, Matsuhashi & Strominger, 1966; Izaki & Strominger, 1968). If these enzymes that recognize acyl-D-alanyl-D-alanine also recognize penicillin, then it seems possible that vancomycin, itself specific for the same peptide sequence, would also accept penicillin as an analogue.

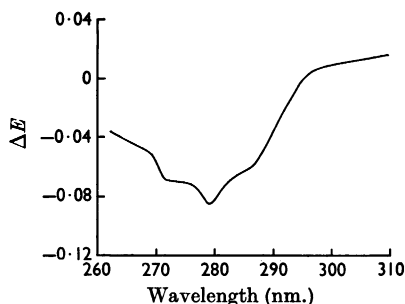

Fig. 8. Differential absorption curve given by a mixture of vancomycin and benzylpenicillin. The sample cell (1mm. light-path) contained a mixture of vancomycin hydrochloride (1·7mM) and sodium benzylpenicillin (16·8mM). The two cells in the reference beam contained the same solutions separately.

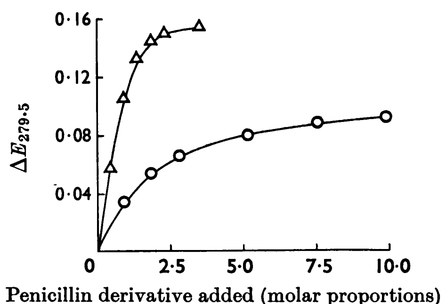

Fig. 9. Differential absorption of vancomycin during addition of penicillin derivatives. The sample cell contained vancomycin, and measured volumes of the penicillin solution were added. Cells in the reference beam contained the same solutions separately. ○, Vancomycin concentration 1·7mM, 1mm.-light-path cell, sodium benzylpenicillin; △, vancomycin concentration 0·17mM, 10mm.-light-path cell, sodium benzylpenicilloate.

The u.v. absorption of vancomycin solution was followed during the addition of sodium benzylpenicillin. The differential absorption curve obtained is given in Fig. 8 and a titration curve measured at 279·5nm. in Fig. 9. The differential absorption curve is not the same as for the peptides described above, but there is clearly some association between the two antibiotics. When penicillin was replaced by sodium phenylacetate or by 6-aminopenicillanic acid, representing the two parts of the penicillin molecule, no differential spectrum was seen. On the other hand, a crystalline sample of sodium benzylpenicilloate gave a differential spectrum and titration curve very similar to those for benzylpenicillin, although the number of moles required to give maximum effect was smaller (Fig. 9).

Combination of vancomycin with sodium benzylpenicilloate was also observed by paper electrophoresis. A sample (0·1μmole) of vancomycin was mixed with sodium benzylpenicilloate (0·3μmole) and subjected to electrophoresis in sodium acetate buffer (0·01M, pH4·6). Free vancomycin moved 4·7cm. towards the cathode, but in the mixed sample it had largely disappeared, being replaced by a new spot at 1·8cm. This spot and free sodium benzylpenicilloate (anionic, 7·1cm.) were also detected by the reagent of Awe, Reinecke, Thum, Neuwald & Ulex (1954).

DISCUSSION

Combination between vancomycin and mucopeptide precursors was first reported by Chatterjee & Perkins (1966*a*). Experiments at that time suggested that no reaction occurred between the two substances *in vitro*, but it later transpired that an excessively dilute vancomycin solution had been used in error, leading to an incorrect result. It is now clear that vancomycin combines readily with complete mucopeptide precursors, as shown by chromatography and also by examination of differential absorption spectra.

The latter method has facilitated a study of the structures that vancomycin will accept and allowed some dissociation constants to be calculated. Thus for the complex between vancomycin and the precursor from *C. poinsettiae* the dissociation

constant was 6×10^{-7} M, and it seems reasonable that formation of such compounds should account for the action of the antibiotic, though at present there is no direct evidence. The smallest molecule found to combine was acetyl-D-alanyl-D-alanine, and all species that reacted contained the same *C*-terminal dipeptide. Removal of one alanine residue, or changing the configuration of either, completely prevented combination. Apparently the free carboxyl group was also required, since esterification with a methyl group also produced an inactive compound. The influence of additional residues at the *N*-terminus is not so easy to define. L-Alanyl was more effective than D-alanyl, and acetyl-L-alanyl-D-alanyl-D-alanine formed a compound with vancomycin having the lowest dissociation constant apart from the complexes with the natural mucopeptide precursors.

The observed specificity of vancomycin for the same *C*-terminal peptide sequence as that which occurs in mucopeptide precursors, and nowhere else in Nature so far as is known, would be a remarkable coincidence if indeed it were unconnected with the mode of action of the drug. Vancomycin may be envisaged to act as follows. On entering the cell it attaches itself to the mucopeptide precursor molecules but does not prevent the loss of UMP and transphosphorylation to the lipid intermediate (Struve, Sinha & Neuhaus, 1966; Matsuhashi *et al.* 1967; Chatterjee *et al.* 1967). Small amounts of the lipid intermediate from *S. aureus* (prepared in the presence of vancomycin), containing *N*-acetylglucosaminyl-*N*-acetylmuramyl-pentapeptide, were apparently able to add [^{14}C]glycine to the ϵ-amino group of the lysine residue in the continued presence of antibiotic (Matsuhashi *et al.* 1967). However, in a similar system from *M. lysodeikticus*, where the added substrate was *N*-acetylglucosaminyl-*N*-acetylmuramyl(pentapeptide)-pyrophospholipid, addition of [^{14}C]glycine to the α-carboxyl group of the D-glutamic acid residue was 73% inhibited by the presence of vancomycin (143μg./ml., 1·6moles/mole of the substrate) (Katz, Matsuhashi, Dietrich & Strominger, 1967). This inhibition was perhaps because each pentapeptide chain carried a vancomycin molecule, which prevented the approach of the enzyme that attaches the glycine residue.

The presumed presence of vancomycin on the pentapeptide chains attached to the lipid intermediate apparently prevents subsequent transfer of mucopeptide to the growing polysaccharide chains (Matsuhashi *et al.* 1967; Chatterjee *et al.* 1967). Since relatively low concentrations of vancomycin are needed for this effect (e.g. mucopeptide synthesis by preparations of *S. aureus* was inhibited about 80% by 10μg. of vancomycin/ml., 0·15mole/mole of substrate; Matsuhashi *et al.* 1967), it seems likely that the transglycosylating enzyme becomes blocked by the vancomycin-bearing lipid intermediate, so that it is no longer available for further synthesis. Alternatively, preformed polysaccharide with non-cross-linked side-chains may be the best acceptor for vancomycin, the presence of which prevents further growth of polysaccharide.

Since vancomycin and ristocetin combine with peptide sequences ending in acyl-D-alanyl-D-alanine, it might be expected that they would inhibit transpeptidases involved in the cross-linking of newly formed mucopeptide, or the D-alanine carboxypeptidase of *E. coli* that removes the terminal residue from the pentapeptide. Tipper & Strominger (1968) concluded, from rather indirect evidence, that, in *S. aureus* even in the presence of vancomycin, 'the previously existing monomer fraction (i.e. non-cross-linked mucopeptide) was utilized to form a cross-linked peptidoglycan'. From the present work, however, it seems most probable that vancomycin would attach itself to the -D-alanyl-D-alanine terminus of the monomer fraction, thus increasing the molecular weight considerably and accounting for the apparent decrease in the proportion of monomer fraction.

The results with the D-alanine carboxypeptidase of *E. coli* B were more clear-cut. Izaki, Matsuhashi & Strominger (1968) incubated cell-free preparations from *E. coli* B with UDP-*N*-acetylmuramyl-L-alanyl-D-glutamyl-*meso*-diaminopimelyl-D-[^{14}C]alanyl-D-[^{14}C]alanine and studied the incorporation of label into mucopeptide and the liberation of free D-alanine. In the presence of 8μg. of vancomycin/ml. (0·25mole/mole of substrate used) liberation of free alanine was decreased by 30%, and with 83μg./ml. (2·6moles/mole) inhibition was 82%. Ristocetin (83μg./ml.) produced 43% inhibition. Evidently the D-alanine carboxypeptidase specific for the pentapeptide was inhibited by these antibiotics, as expected from the complex-formation with the substrate. According to Izaki & Strominger (1968) the purified D-alanine carboxypeptidase I of *E. coli* B, specific for the pentapeptide, was only 50% inhibited by 50μg. of vancomycin/ml. (3moles/mole of substrate). The apparent relative insensitivity of the purified enzyme system merits further investigation.

Tipper & Strominger (1965) suggested that penicillin may act as an analogue of acyl-D-alanyl-D-alanine, acylating and thus blocking the D-alanine transpeptidase of mucopeptide cross-linking. If this enzyme recognizes acyl-D-alanyl-D-alanine and can accept penicillin as an analogue, it seems possible that vancomycin, shown by the present results to recognize the same peptide fragment, would also accept penicillin as an analogue. The observation that benzylpenicillin yielded a differential

spectrum with vancomycin may offer some support for this idea. On the other hand, sodium benzylpenicilloate, which is not antibiotically active, seemed to combine even more readily with vancomycin, suggesting that it was a better analogue of acyl-D-alanyl-D-alanine. This paradox might be resolved by assuming that the sensitive enzymes, such as the transpeptidase of mucopeptide cross-linking, first accept penicillin and are then acylated by it at their active sites, so that they can no longer function. The dissociable complex of enzyme with preformed penicilloate would not be sufficiently stable to inhibit the enzyme except at high concentrations.

Products of chemical modification of vancomycin that were antibiotically active also combined with mucopeptide precursors. The fact that vancomycin could be acetylated or formylated and yet combine suggests that the free amino group may not be directly involved in antibiotic action. So little is known of vancomycin structure, however, that it is not possible to envisage the chemical nature of the region that accepts acyl-D-alanyl-D-alanine, except to say that phenolic groups must be involved.

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