Biochem. J. (1970) **116**, 83–92
Printed in Great Britain

The Preparation of Iodinated Vancomycin and its Distribution in Bacteria Treated with the Antibiotic

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(*Received* 13 *August* 1969)

Vancomycin was radioactively labelled by iodination with ^{125}I. Iodinated vancomycin was only a little less potent as an antibiotic than vancomycin itself. It was shown, both by chromatography and differential absorption measurements, to combine with acyl-D-alanyl-D-alanine residues. Radioactive vancomycin was used to follow the fate of the antibiotic in bacteria that had been subjected to the least concentration required to inhibit growth. Most of the radioactivity was in the cell walls, although some was found in the membrane fraction. The latter proportion increased during longer incubations with the antibiotic. Pre-formed protoplasts adsorbed very little vancomycin. Mg^{2+} removed labelled vancomycin from the mucopeptide of *Bacillus licheniformis*, but had little effect on the antibiotic adsorbed on *Micrococcus lysodeikticus*, either *in vivo* or on previously isolated cell walls. Specific peptide was shown to compete with cell walls for vancomycin and it also extracted from cell-wall samples the labelled compound that had been adsorbed on *M. lysodeikticus* living cells.

Vancomycin, an antibiotic obtained from cultures of *Streptomyces orientalis*, is known to inhibit the biosynthesis of the cell-wall mucopeptide in cell-free systems (Anderson, Matsuhashi, Haskin & Strominger, 1965, 1967) and to cause the accumulation of UDP-*N*-acetylmuramyl-peptide precursors in whole cells (Reynolds, 1961, 1966; Jordan, 1961). Best & Durham (1964) showed that at concentrations of less than 2μg/mg dry wt. of cells vancomycin inhibited cell-wall synthesis in *Bacillus subtilis* W 23 and that Mg^{2+} alleviated the inhibition. Later, they treated both whole cells and isolated cell walls of the same organism with high concentrations of vancomycin and showed that the antibiotic was adsorbed, as indicated by a fall in E_{280} of the supernatant. This adsorption was also greatly decreased by the presence of bivalent cations (Mg^{2+}, Mn^{2+}, Ca^{2+} and Fe^{2+}) or of polylysine or spermine (Best & Durham, 1965). Sinha & Neuhaus (1968) found that the isolated cell walls of *Micrococcus lysodeikticus* (1mg) absorbed 95% of vancomycin from solution (1mg/ml) in pH 7.8 buffer, whereas in the presence of 10mM-Mn^{2+} 2mg of cell walls was required to produce a maximum absorption of 80%. Thus in this species bivalent cation could be regarded as approximately halving the number of sites available for vancomycin adsorption. It is noteworthy that in the absence of Mn^{2+} the cell walls of *M. lysodeikticus* adsorbed almost their own weight of vancomycin.

Results obtained by these methods were limited by the fact that relatively high concentrations of vancomycin had to be used, far in excess of the minimum inhibitory concentration for bacterial growth. Also they provided no information about the fate of vancomycin that was adsorbed by living cells. These problems could be overcome by the use of radioactive vancomycin, but this is not commercially available. The work of Johnson (1962) and Marshall (1965) indicated that the vancomycin molecule contained phenols and chlorophenols, so that iodination might well be feasible. We found that vancomycin could be easily iodinated by a technique similar to that used for tyrosine residues in protein molecules (McFarlane, 1958). The resulting compound retained its antibiotic activity and could thus be used to follow the distribution in treated cells.

METHODS

Iodination. Vancomycin hydrochloride (Vancocin HCl) was kindly given by Eli Lilly and Co. Ltd., Basingstoke, Hants. A sample (32mg) was dissolved in water (2ml) and 20μl of M-NaOH was added, causing some turbidity. In another vessel, to 0.6ml of 33mM-ICl in M-NaCl solution at pH 1 (McFarlane, 1963) was added 60μl of 12.5M-NaOH, followed by 1ml of 0.1M-$NaHCO_3$–Na_2CO_3 buffer, pH 9.5. This solution was added to 0.2mCi of [^{125}I]iodide (iodide carrier-free for protein iodination; IMS3; The Radiochemical Centre, Amersham, Bucks., U.K.) and the radioactive solution was added to the vancomycin with stirring, over a period of about 1min, so that the vancomycin remained in excess as long as possible. The mixture was diluted with about 5ml of water and 0.2ml each of KI (0.1M) and KIO_3 (16.7mM) were added, and the solution was acidified with HCl. The iodine released was extracted into CCl_4 under an efficient fume-hood, and the

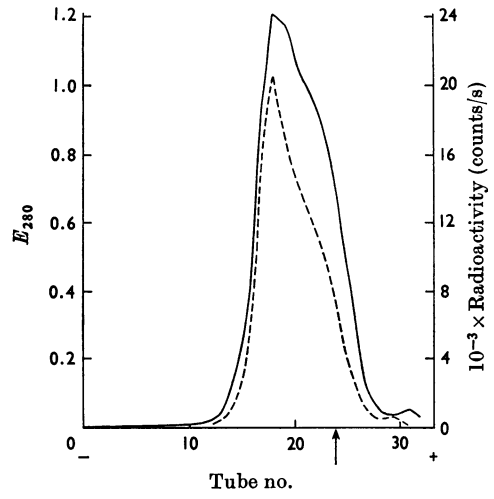

Fig. 1. Continuous-flow paper electrophoresis of iodinated vancomycin. The electrolyte was 0.1 M-formic acid. The arrow indicates the point of application of the sample. ——, Radioactivity of samples measured directly in a well-counter; – – – –, E_{280}.

CCl_4 was immediately decolorized by shaking with a solution of $Na_2S_2O_3$. The aqueous solution of radioactive vancomycin was purified by use of an apparatus for continuous-flow paper electrophoresis (Beckman Instruments Inc., Spinco division, model CP). The electrolyte was 0.1 M-formic acid. A constant current of 40 mA was passed and the applied potential was approx. 500 V. Fractions were collected, the tubes being changed automatically every 12 h. The position of the product was determined by measurement of radioactivity and E_{280}. A single skewed peak was obtained, centred on a tube slightly towards the cathode from the point of application (Fig. 1). Any residual I^- was removed, since it moved towards the anode. The pooled samples were concentrated to a final volume of 10 ml. The vancomycin concentration (assuming that the E_{280} was the same as for vancomycin hydrochloride) was 3.5 mg/ml and the radioactivity was 1.17×10^5 counts/s per ml (counting efficiency 46%). This means that about 35% of the added radioactivity appeared in the product.

Non-radioactive iodinated vancomycin was also prepared by the same method. Freeze-dried samples were found to contain 4.16% I, 3.16% ash. This corresponds to 1 g-atom of I for every 3160 g of volatile matter, a value close to the molecular weight proposed for vancomycin (for discussion of this point see Perkins, 1969). In other experiments we found that by increasing fivefold the molar proportion of ICl used in the reaction a sample of iodinated product could be obtained with 7.21% I, 4.25% ash, corresponding to 1 g-atom of I for 1690 g of volatile matter. This procedure was relatively inefficient in utilization of iodine, and it was therefore not employed for radioactive work.

Growth of organisms. *M. lysodeikticus* N.C.T.C. 2665 was grown at either 30°C or 34°C in Hedley–Wright broth containing 1% (w/v) of glucose. *B. subtilis* W23 was transferred from a spore suspension into medium 0.5 CY (Novick, 1963). For experiments on vancomycin inhibition cells were then transferred to the 'glucose–salts' medium of Best & Durham (1964), since preliminary experiments indicated that when bacteria growing in 0.5 CY medium were treated with vancomycin lysis occurred so rapidly as to make the system unmanageable. The density of cell suspensions was measured in cuvettes of 1 cm light-path on a Spekker absorptiometer (Hilger and Watts) with neutral filter. *Staphylococcus aureus* strain H, *Corynebacterium poinsettiae* (N.C.P.P. 177), *Corynebacterium sepedonicum* (N.C.P.P. 378), *Corynebacterium tritici* (N.C.P.P. 471), *Corynebacterium insidiosum* (N.C.P.P. 1110) and *Corynebacterium flaccumfaciens* var. *auranticum* (N.C.P.P. 558) were also used.

Preparation of cell walls. The bacteria were harvested by centrifuging, washed in 50 mM-phosphate buffer, pH 7.0, and resuspended in water to give 20 mg dry wt./ml. An equal volume of glass beads (Ballotini no. 12) was added and the cells were disrupted in the cold in an oscillatory homogenizer (B. Braun, Melsungen, Germany) for 2–3 min. The mixture was heated at 60°C for 10 min in an attempt to inactivate lytic enzymes. It was then cooled and, after the glass beads had been filtered off on a sinter, centrifuged at 12 000*g* for 20 min at 5°C. The crude cell walls were resuspended in water and centrifuged at 1000*g* for 10 min at 5°C to remove any unbroken cells. The supernatant was then centrifuged at 38 000*g* for 5 min. The cell walls were washed with M-NaCl and then finally resuspended in 50 mM-phosphate buffer, pH 7.6. Trypsin was added (1 mg/ml) and incubation was continued overnight at 37°C. The purified cell walls were recovered by centrifuging at 12 000*g* for 30 min at 5°C and washed once with water.

Protoplasts and protoplast membranes. Cells were harvested by centrifuging and washed with 10 mM-phosphate buffer, pH 7. They were then resuspended in 0.1 M-ammonium acetate buffer, pH 6.0, containing sucrose (1.0 M) and $MgCl_2$ (10 mM or 20 mM). Lysozyme (BDH Chemicals Ltd., Poole, Dorset, U.K.) was added to a final concentration of 0.1 mg/ml and the suspension was incubated at 35°C for 1 h, or sometimes overnight. It was then centrifuged at 8000*g* for 10 min. The protoplasts were washed in buffer–Mg^{2+}–sucrose and then burst by resuspending them in the original volume of ice-cold 10 mM-tris–HCl buffer, pH 7.8, containing $MgCl_2$ (10 mM). A trace of deoxyribonuclease was added and the suspension was shaken for 15 min and allowed to reach room temperature. It was then centrifuged at 28 000*g* for 20 min. The membrane pellet was washed once in buffer before being resuspended for measurement of radioactivity.

Measurement of radioactivity. The radioactivity of 1 ml samples was measured either in a well-counter (Isotope Developments Ltd., Aldermaston, Berks., U.K.; counting efficiency for ^{125}I, 46%) or in a Packard Autogamma spectrometer (efficiency for ^{125}I, 39%).

Complex-formation between antibiotic and peptides. This was observed qualitatively on paper chromatograms and quantitatively by differential spectroscopy as described before (Perkins, 1969). The chromatographic solvents were ethanol–M-ammonium acetate (5:2, v/v) and isobutyric acid–aq. 0.5 M-NH_3 (5:3, v/v).

Peptides. Diacetyl-L-lysyl-D-alanyl-D-alanine, tyrosyl-D-glutamyl-L-lysyl-D-alanyl-D-alanine and iodotyrosyl-

D-glutamyl-L-lysyl-D-alanyl-D-alanine were synthesized by the solid-phase method (Merrifield, 1963; Marshall & Merrifield, 1965).

Cell walls. A sample of the purified mucopeptide fraction of the cell walls of *Bacillus licheniformis* 6346 was kindly given by Dr R. C. Hughes. The cell walls and mucopeptide of *C. poinsettiae* were prepared by Dr Teresa Diaz-Mauriño. The cell walls contained 50% mucopeptide, the rest being polysaccharide.

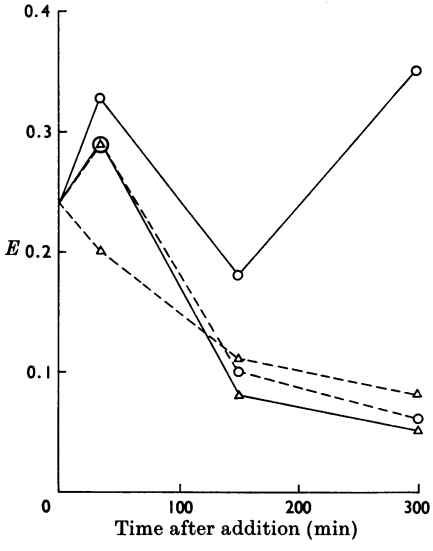

Fig. 2. Growth inhibition of *B. subtilis* W23 with vancomycin and iodinated vancomycin (7.5% I). The bacteria were grown in 0.5 CY medium and a small inoculum was transferred to glucose–salts medium. When the extinction was 0.242, samples (20ml) were taken and antibiotic was added. Shaking at 35°C was continued and the extinctions were measured. ○, Iodinated vancomycin; △, vancomycin. ——, 0.25 μg/ml; – – – –, 0.5 μg/ml.

RESULTS

Iodinated vancomycin. Vancomycin was made radioactive by iodination with ^{125}I. The antibiotic potency of the product was tested against *C. poinsettiae* on agar plates as described by Perkins (1969) or in some experiments the concentration required to inhibit exponential growth of *B. subtilis* W23 in glucose–salts medium was measured. The iodinated vancomycin, whether containing 4% or 7% of iodine, was almost as inhibitory as the untreated antibiotic. For instance, the effect on *B. subtilis* of the product containing 7% I can be seen in Fig. 2. Thus cells continued to grow for a short time after addition of 0.25 or 0.50 μg of iodinated vancomycin/ml, but then growth stopped and lysis ensued. At the lower concentration recovery of growth occurred after some lapse of time. In other experiments it was found that below 0.25 μg/ml even vancomycin hydrochloride would not halt the growth of *B. subtilis* W23.

It is known that vancomycin will form stable complexes with mucopeptide precursors or peptide analogues that terminate in acyl-D-alanyl-D-alanine (Perkins, 1969). On chromatograms developed in isobutyric acid–ammonia or ethanol–ammonium acetate the complexes ran more slowly than free vancomycin. When similar experiments were performed with iodinated vancomycin and appropriate peptides, the complexes, easily detected by radioautography, again ran more slowly than the parent compounds (Table 1). It was noticeable that, although the iodinated vancomycin hardly separated from vancomycin, its complexes with nucleotide or peptide always moved further on the chromatograms than those with the untreated antibiotic.

Samples of iodinated vancomycin (4% and 7% I) were titrated with a synthetic sample of diacetyl-L-lysyl-D-alanyl-D-alanine (Perkins & Nieto, 1969) and

Table 1. *Chromatography of vancomycin, iodinated vancomycin and complexes with peptides*

Whatman no. 3MM paper was washed extensively with M-ammonium acetate and then with water and dried before use. Chromatograms were developed with solvent for 18h. Considerable variation in R_F values was observed on different occasions, but the order of spots was constant. With unwashed paper the complex of vancomycin and UDP-*N*-acetylmuramyl-pentapeptide sometimes remained on the origin even when the solvent was ethanol–ammonium acetate.

	$R_{vancomycin}$	
	Ethanol–ammonium acetate	Isobutyric acid–ammonia
(1) Iodinated vancomycin (4% I)	1.02	1.01
(2) UDP-*N*-acetylmuramyl-L-alanyl-D-isoglutamyl-L-lysyl-D-alanyl-D-alanine	0.92	0.31
(3) Tyrosyl-D-glutamyl-L-lysyl-D-alanyl-D-alanine	0.97	1.79
(4) Iodotyrosyl-D-glutamyl-L-lysyl-D-alanyl-D-alanine	0.97	2.06
(5) Vancomycin + (2)	0.50	0
(6) (1) + (2)	0.70	0.16
(7) Vancomycin + (3)	0.68	0.54
(8) (1) + (3)	0.79	0.74
(9) Vancomycin + (4)	0.68	0.54
(10) (1) + (4)	0.80	0.68

the differential absorption was measured in a double-beam photoelectric absorptiometer. The results (Fig. 3) show that complex-formation with iodinated vancomycin (4% I) was quantitatively comparable with that observed previously with vancomycin (Perkins, 1969). However, the antibiotic containing 7% I combined with only one molecule of peptide for each 3200 of molecular weight, instead of the two molecules of peptide observed with vancomycin.

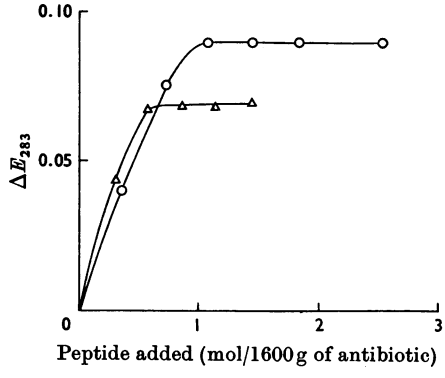

Fig. 3. Titration of iodinated vancomycins with diacetyl-L-lysyl-D-alanyl-D-alanine. The ordinate represents the decrease in extinction of vancomycin at 283nm (a minimum in the differential curve). ○, Iodinated vancomycin (4% I), 0.11mM; △, iodinated vancomycin (7.5% I), 0.17mM.

Uptake of iodinated vancomycin by bacteria. (a) M. lysodeikticus. The first experiments were performed with stationary-phase *M. lysodeikticus*. The cells were harvested and resuspended in broth at 1mg dry wt./ml. Iodinated vancomycin was added to a concentration of 10μg/ml and the flask was shaken for 15min. In preliminary experiments samples were withdrawn at intermediate times and immediately cooled and centrifuged. Even after 3min uptake of radioactivity was already complete. After incubation with antibiotic part of the culture was used for the preparation of cell walls, and another portion was made 10mM with respect to magnesium chloride and shaken for a further 30min. This portion was also used for isolation of cell walls. The result of such an experiment is given in Table 2. Contrary to what might have been expected from the work of Best & Durham (1965) with *B. subtilis*, the addition of 10mM-magnesium chloride had no effect on the uptake of vancomycin, at least at these low concentrations. Table 2 also shows that 10mM-Mg^{2+} removed only a trace of the iodinated vancomycin that had been adsorbed on the cell walls *in vivo*. The purified salt-extracted and trypsin-digested cell-wall preparations still retained 75% of the radioactivity in the absence of added Mg^{2+}, and 68% when 10mM-Mg^{2+} was present. Thus iodinated vancomycin, like the untreated antibiotic, is mainly adsorbed on the cell walls of viable stationary-phase cells, although a portion (some 10–15%) must have been in some other part of the cell.

In view of the contrast with the results of Best & Durham (1965) on the inhibitory effect of Mg^{2+} on

Table 2. *Distribution of iodinated vancomycin in stationary-phase cells of* M. lysodeikticus

The cells were grown in broth overnight at 30°C in a shaken flask. After being harvested and washed in water the cells were resuspended in fresh broth (200ml) to give an extinction equivalent to 1mg dry wt./ml. Then iodinated vancomycin, ^{125}I-labelled, was added to a final concentration of 10μg/ml. The culture was shaken at 30°C for 15min. At this time 100ml was harvested. To the remainder $MgCl_2$ was added (10mM final concn.) and shaking was continued for 30min before harvest. The cells were washed and samples were taken for measurement of radioactivity at the various stages of cell-wall preparation.

	No Mg^{2+} added		10mM-Mg^{2+} added	
	Radioactivity (counts/s)	% of radioactivity in washed cells	Radioactivity (counts/s)	% of radioactivity in washed cells
Total culture (100ml)	17100		17100	
Supernatant and wash	5010		4656	
Washed cells (by difference)	12090 (71% uptake)	100	12444 (73% uptake)	100
Unbroken cells	600	5.0	870	7.0
Wall supernatant and wash	1168	9.7	1912	15.4
M-NaCl wash	44	0.4	—	—
10mM-$MgCl_2$ wash	—	—	28	0.2
M-NaCl wash	43	0.4	60	0.5
Trypsin supernatant and wash	322	2.7	338	2.7
Final walls	9126	75.5	8486	68.2
Total recovery from washed cells		93.7		94.0

Table 3. *Adsorption of vancomycin by isolated cell walls of* M. lysodeikticus: *effect of* Mg^{2+} *and sodium dodecyl sulphate*

Vancomycin hydrochloride (0.9mg) was dissolved in water (6ml) and mixed with freeze-dried cell-wall preparations (2mg). The tubes were centrifuged, the supernatant was removed and E_{280} was measured. The cell-wall residue was extracted first with 10mM-$MgCl_2$ (6ml) and then with 0.1% (w/v) sodium dodecyl sulphate (6ml).

	Wall preparation 1			Wall preparation 2		
	Vancomycin (μg)	% adsorbed	% extracted	Vancomycin (μg)	% adsorbed	% extracted
Supernatant	110	88		170	81	
Adsorbed on walls	790			730		
10mM-$MgCl_2$ extract	94		12	102		14
Residue	696			628		
Dodecyl sulphate extract	146		21	141		22
Residue	550			487		

Table 4. *Distribution of iodinated vancomycin added to growing cultures of* M. lysodeikticus

The cells were growing in broth at 34°C. Iodinated vancomycin, ^{125}I-labelled, was added to a final concentration of 10μg/ml when the cell concentration had reached about 0.3mg dry wt./ml. Samples were taken at the times shown in Fig. 4 and the cells used for preparation of cell walls (Expt. A) or protoplasts and hence cytoplasmic membranes (Expt. B). In Expt. A the cells adsorbed 64% of the radioactive antibiotic, and in Expt. B 58%.

	Percentage of adsorbed radioactivity			
	Expt. A	Expt. B		
Time after addition of vancomycin	90min	Sample 1 5min	Sample 2 60min	Sample 3 235min
Unbroken cells	4.3			
M-NaCl washes	5.5			
Trypsin supernatant and wash	4.0			
Purified cell walls	23.5			
Membrane fraction (by differential centrifuging)	34.6			
Soluble fraction	13.5			
Lysozyme digest in M-sucrose, supernatant (solubilized cell walls)		66.3	56.3	22.2
Protoplast membranes		8.2	16.6	52.3
Protoplast, soluble fraction		6.8	9.8	11.4
Total recovery	85.4	81.3	82.7	85.9

vancomycin absorption, their procedure was followed in an experiment with isolated cell walls of *M. lysodeikticus* and vancomycin hydrochloride. Table 3 shows that in the presence of vancomycin hydrochloride (150μg/ml), cell walls (0.33mg/ml) adsorbed about 130μg of antibiotic. This compares closely with the results for *B. subtilis* (Best & Durham, 1965). However, extraction with 10mM-Mg^{2+} removed very little of the adsorbed vancomycin, thus confirming the observation with cell walls prepared from *M. lysodeikticus* labelled *in vivo* with iodinated vancomycin (Table 2). It seemed possible that the vancomycin adsorbed on the cell walls might be attached by van der Waals forces and might therefore be extracted by sodium dodecyl sulphate. However, only about 20% of the adsorbed antibiotic was extracted from the cell walls of *M. lysodeikticus* (Table 3), although this may well be species-dependent as with Mg^{2+} extraction.

The availability of labelled vancomycin offered the possibility of examining the fate of the antibiotic in conditions where growing cells were only just inhibited. It might be expected that the antibiotic would then be found at the site where it exerts its specific action. Exponentially growing cultures of *M. lysodeikticus* were treated with iodinated vancomycin in such concentrations that growth was just halted, but extensive lysis did not occur. Samples were removed from the culture at various times and the cells were used for the preparation of cell walls or isolated cytoplasmic membranes (Table 4 and Fig. 4). The most striking observation was that, at vancomycin concentrations that were just inhibitory for growth, the proportion of antibiotic found in the

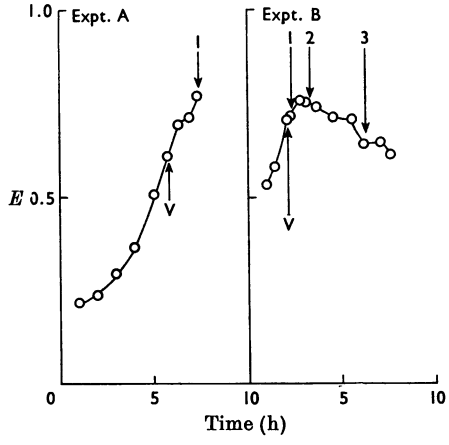

Fig. 4. Addition of iodinated vancomycin (4% I) to cultures of *M. lysodeikticus*. Samples taken at the points arrowed (1–3) were fractionated and their radioactivities measured as shown in Table 4. Antibiotic (10 μg/ml) was added at the point labelled V.

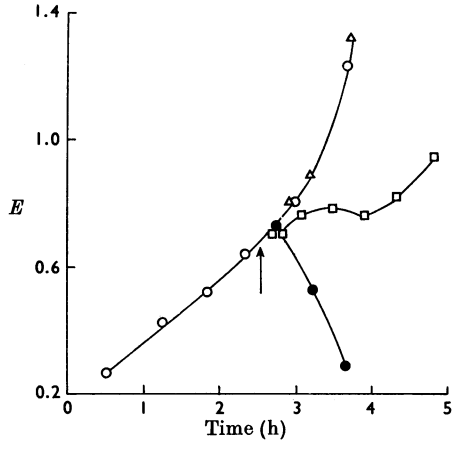

Fig. 5. Effect of iodinated vancomycin (4% I) on growth of *B. subtilis* W23. The bacteria were multiplying in glucose–salts medium at 35°C; antibiotic was added at the arrow to three samples, and shaking was continued. Concentration of iodinated vancomycin: ○, none; △, 0.25 μg/ml; □, 0.5 μg/ml; ●, 1.0 μg/ml.

cell-wall fraction initially (Expt. B, sample 1) was almost as high as in stationary-phase cells (Table 2). As time elapsed, however, vancomycin disappeared from the cell-wall fraction and accumulated in the protoplast-membrane fraction.

(*b*) *B. subtilis*. *B. subtilis* W23 is very sensitive to vancomycin (Best & Durham, 1964) and also to iodinated vancomycin (Figs. 2 and 5). The apparent greater sensitivity observed in Fig. 2 was presumably due to the lower cell density at the point of vancomycin addition in that experiment. We could not obtain cell-wall preparations after inhibition with minimum inhibitory concentration of iodinated vancomycin, because the cell walls lysed during purification in spite of the 10 min period of heating at 60°C included in the procedure. Thus in a typical experiment, in which *B. subtilis* cells growing exponentially in glucose–salts medium were incubated for 15 min with 1 μg of iodinated vancomycin/ml, the cells adsorbed 51% of the added radioactivity. Of the adsorbed radioactivity only 6% appeared in the final cell walls but 35% was in the non-sedimentable portion (23 000*g*) of the broken cells and 31% was in the trypsin-digest soluble fraction. However, this dissolution was not due to trypsin, since almost as much became soluble in a control digest to which no trypsin had been added.

The distribution of adsorbed iodinated vancomycin in *B. subtilis* W23 was therefore followed during preparations of protoplasts and cell membranes by a procedure similar to that used for *M. lysodeikticus*. The extinction of growing cultures was measured and samples were taken for protoplast preparation 12 min after addition of iodinated vancomycin (1 μg/ml), when a decline of growth rate was just detectable. The distribution of radioactivity in the cell fractions is shown in Table 5. A large proportion of the adsorbed iodinated vancomycin was in the cell-wall fraction. Nevertheless, as with *M. lysodeikticus*, 10% or more of the antibiotic was in the protoplast-membrane fraction. In Expt. 2 the membrane component was 25% of the whole, a value reminiscent of those seen with *M. lysodeikticus* incubated for a longer period with iodinated vancomycin (Table 4).

Adsorption of iodinated vancomycin by preformed protoplasts. Cells of *M. lysodeikticus* growing exponentially at 35°C (about 1 mg dry wt./ml) were treated with lysozyme to prepare protoplasts, stabilized with 20 mM-Mg^{2+} and M-sucrose. The washed protoplasts were incubated in the same medium with iodinated vancomycin (4% I) for a shorter or longer period. The uptake of radioactivity and the proportion of radioactivity that remained in the membrane fractions after the protoplasts had been burst were measured (Table 6). The radioactivity in the membranes represented a very small uptake of vancomycin, much smaller than in membranes isolated from whole cells pretreated with iodinated vancomycin (Table 4).

Extraction of adsorbed iodinated vancomycin with trichloroacetic acid. Chatterjee & Perkins (1966) first reported that, from bacteria inhibited with vancomycin, 25% (w/v) trichloroacetic acid extracted not only UDP-*N*-acetylmuramyl-pentapeptide but also a complex of the same nucleotide with vancomycin. Since it is now known that the complex forms on

Table 5. *Distribution of iodinated vancomycin added to growing cultures of* B. subtilis *W* 23

The cells were growing in glucose–salts medium at 34°C. Iodinated vancomycin, ^{125}I-labelled, was added to a final concentration of 1 μg/ml when the cell concentration had reached about 0.4 mg dry wt./ml. After 12 min the cells were harvested, washed in ice-cold 10 mM-phosphate buffer, pH 7, and used for preparation of protoplasts and hence cytoplasmic membranes. The proportion of radioactive antibiotic retained by washed cells was as follows: Expt. 1, 51%; Expt. 2, 42%; Expt. 3, 46%. These were separate experiments performed on different occasions.

	Percentage of adsorbed radioactivity		
	Expt. 1	Expt. 2	Expt. 3
Lysozyme digest in M-sucrose, supernatant (solubilized cell walls)	72.0	58.7	82.2
Protoplast membranes	10.8	25.3	11.2
Protoplast, soluble fraction	13.0	10.2	5.6
Total recovery from washed cells	95.8	94.2	99.0

Table 6. *Preformed protoplasts treated with iodinated vancomycin*

M. lysodeikticus cells in exponential growth phase (approx. 0.5 mg dry wt./ml) were centrifuged and resuspended for protoplast formation at 25 mg dry wt./ml as follows. Expt. A: they were suspended in 0.1 M-ammonium acetate buffer, pH 6, containing M-sucrose and 20 mM-$MgCl_2$ and incubated with lysozyme (100 μg/ml) for 2 h at 35°C. Expt. B: they were suspended in 0.1 M-phosphate buffer, pH 7, containing M-sucrose, 20 mM-$MgCl_2$ and 56 mM-glucose and incubated with lysozyme (100 μg/ml) for 30 min at 35°C; the protoplasts were recovered by centrifuging, washed and resuspended in the same volume of the same buffered sucrose used for digestion; iodinated vancomycin (4% I) was added (10 μg/ml) to separate portions (2 ml), which were incubated at 35°C for different periods. Expt. C: glutamic acid, alanine, glycine and lysine were added (each at 100 μg/ml) during the final incubation with iodinated vancomycin. The protoplasts prepared as in Expt. B were washed twice with buffered sucrose and then the residue was resuspended in 10 mM-tris–HCl buffer, pH 7.8, containing 20 mM-$MgCl_2$ and a trace of deoxyribonuclease. The membranes were recovered by centrifuging (28 000*g* for 10 min) and washed in the same buffer. Radioactivity of all fractions was measured.

	Percentage of total counts in suspension					
	Expt. A		Expt. B		Expt. C	
	10 min incubation	75 min incubation	No incubation	90 min incubation	No incubation	90 min incubation
Sucrose supernatant 1	76.0	75.8	70.6	66.8	55.6	74.0
Sucrose supernatant 2	19.0	14.0	19.4	25.7	26.3	18.7
Sucrose supernatant 3	2.7	3.4	4.6	7.6	12.9	5.2
Burst protoplast supernatant	1.0	1.1	1.1	1.1	2.2	1.4
Tris buffer wash	0	0.3	0	0	0	0
Membrane fraction	0.6	1.9	0.9	0.3	1.2	0.7
Recovery	99.3	96.5	96.6	101.2	98.2	100.2

mixing the antibiotic and the nucleotide-pentapeptide (Perkins, 1969) it is clear that these complexes could arise in the extract by simultaneous removal of nucleotide from within the cell and vancomycin from the cell wall, the principal site of vancomycin adsorption.

Bacteria of various Gram-positive species growing in broth were treated with iodinated vancomycin (10 μg/ml) and shaken for a further 90 min as in the procedure for nucleotide accumulation (Chatterjee & Perkins, 1966). The cells were harvested and extracted with trichloroacetic acid at various concentrations and the amount of radioactivity extracted was measured (Table 7). The plant-pathogenic corynebacteria (except *C. insidiosum*) adsorbed a somewhat lower proportion of the radioactive vancomycin than the cocci. Regardless of species, a large proportion of the adsorbed antibiotic was extracted by trichloroacetic acid. Various concentrations of extractant were tested with the two cocci but little difference was observed, apart from a slight reluctance for the antibiotic to pass from *S. aureus* into 5% trichloroacetic acid.

In another experiment *C. poinsettiae* cells were

Table 7. *Iodinated vancomycin adsorbed by growing cells: extraction with trichloroacetic acid*

The bacteria were growing at their optimum temperatures in broth containing 1% of glucose. Iodinated vancomycin was added to a final concentration of 10 μg/ml and shaking was continued for 1.5 h. The bacteria were then harvested and extracted at 2°C with trichloroacetic acid solution for 10 min periods. The radioactivity of the whole culture, the cell supernatant and the extracts was measured.

Organism	mg dry wt./ml at vancomycin addition	% of radioactivity found in cells	Concn. of trichloroacetic acid (%, w/v)	Percentage of adsorbed radioactivity in: First extract	Second extract	Residual cells
C. poinsettiae	0.74	41	25	74	16	
C. sepedonicum	0.90	45	25	72	20	
C. tritici	0.52	32	25	81	6	
C. flaccumfaciens	0.86	42	25	68	14	
C. insidiosum		55	25	83	4	
M. lysodeikticus		57	5	71	10	16
		57	10	78	9	10
		57	25	77	5	6
S. aureus H		74	5	56	25	18
		74	10	79	10	9
		74	25	76	7	2

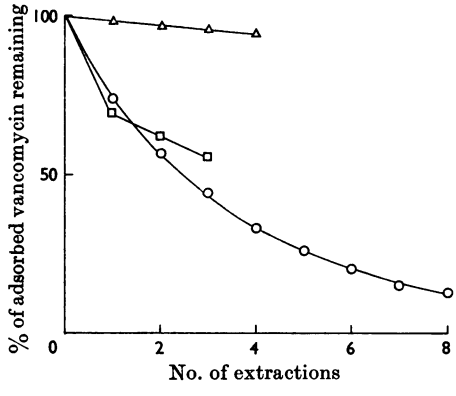

Fig. 6. Extraction of adsorbed iodinated vancomycin with salts or specific peptide. ○, Antibiotic (20 μg/ml) added to mucopeptide of *B. licheniformis* (2 mg in 2 ml of water): 65% was adsorbed. The centrifuged residue was resuspended in 2 ml of salt solution (M-NaCl–10 mM-$MgCl_2$) and the radioactivity measured. After centrifuging the radioactivity of the supernatant was measured and the procedure was repeated. △, Cell walls of *M. lysodeikticus* labelled *in vivo* (experiment of Table 2, no Mg^{2+}): 1.3 mg was suspended in 4 ml of salt solution (M-NaCl–10 mM-$MgCl_2$) and recentrifuged. Radioactivity was measured as before. □, Same cell walls of *M. lysodeikticus*, but sample extracted with diacetyl-L-lysyl-D-alanyl-D-alanine (0.1 mM).

inhibited with iodinated vancomycin (4% I; 25 μg/ml) and chloramphenicol (60 μg/ml) as before (Chatterjee & Perkins, 1966). After incubation for 90 min the cells were extracted either with 25% (w/v) trichloroacetic acid in the cold or with water at 100°C for 10 min. The cells had adsorbed 66% of the radioactive vancomycin. In two extractions cold trichloroacetic acid extracted 87% of the adsorbed antibiotic, whereas hot water removed only 17%. From separate samples of the same cells treated with vancomycin hydrochloride (25 μg/ml) hot water extracted more precursor nucleotide (measured as *N*-acetylhexosamine after 5 min hydrolysis at 100°C in 50 mM-hydrochloric acid) (13.8 μmol/g dry wt. of cells) than did cold trichloroacetic acid (10.7 μmol/g dry wt. of cells). Thus hot-water extraction greatly decreased contamination of extracted nucleotide with vancomycin.

Extraction of adsorbed iodinated vancomycin from cell walls and mucopeptide. Iodinated vancomycin was adsorbed by a sample of the purified mucopeptide of *B. licheniformis* that was then repeatedly extracted with a solution containing M-sodium chloride and 10 mM-magnesium chloride. The progress of extraction was compared with the results for a sample of *M. lysodeikticus* cell walls prepared from cells labelled *in vivo* (Fig. 6). Whereas vancomycin was extracted only very slowly from the cell walls of *M. lysodeikticus*, repeated salt extraction removed almost all the antibiotic from *B. licheniformis* mucopeptide.

Vancomycin is known to form stable complexes with acyl-D-alanyl-D-alanine peptides (Perkins, 1969), and one of these peptides was used to extract iodinated vancomycin from the isolated cell walls of *M. lysodeikticus* labelled *in vivo* (Fig. 6). The initial extraction was comparatively rapid, but after that a steady rate followed, suggesting perhaps that some non-specifically bound antibiotic was extracted first

Table 8. *Competition between cell walls or mucopeptide and synthetic peptide for binding sites on iodinated vancomycin*

To 2ml of suspensions of the purified mucopeptide or walls of the bacteria indicated in the table at the concentration shown were added: Expt. 1: 10μl of a solution of iodinated vancomycin (4% I) containing 1.71mg/ml; Expt. 2: 0.02μmol of N^2,N^6-diacetyl-L-lysyl-D-alanyl-D-alanine; the mixture was shaken for 5min and then iodinated vancomycin was added as in Expt. 1; Expt. 3: iodinated vancomycin and peptide as in Expt. 2 were mixed first and then added to the suspension; Expt. 4: as Expt. 3, but 0.2μmol of peptide was used. The suspensions were shaken for 30min at room temperature, then a 0.5ml sample was pipetted out to measure the radioactivity of the whole system. The 1.5ml remaining was centrifuged at 10000*g* for 10min. The water-clear supernatants were pipetted out and the radioactivity of a 0.5ml sample was measured. The pads were resuspended in 1.5ml of water and their radioactivities measured as well. Both were consistent. The values in the table are the percentages of the radioactivity in the whole system that appeared in the pad after centrifuging.

	Percentage of added radioactivity adsorbed		
Expt. no.	*B. licheniformis* mucopeptide (0.5mg/ml)	*C. poinsettiae* cell walls (1.0mg/ml)	*C. poinsettiae* mucopeptide (0.5mg/ml)
1	67.6	80.6	73.5
2	59.7	73.1	70.5
3	55.0	75.4	65.7
4	33.7	39.6	13.4

and that thereafter exchange occurred with antibiotic bound to D-alanyl-D-alanine termini in the cell wall. The rate of extraction from the cell walls of *M. lysodeikticus* was much greater with specific peptide than with salts.

Competition for vancomycin binding between specific soluble peptide and insoluble cell-wall samples was also examined (Table 8). The simultaneous presence of diacetyl-L-lysyl-D-alanyl-D-alanine (0.1mM) decreased the adsorption of iodinated vancomycin by cell walls or mucopeptide by more than 50%.

DISCUSSION

Just as mucopeptide precursor nucleotides are extracted from cells by cold trichloroacetic acid, so also is vancomycin extracted from the cell wall. This result would explain the earlier observation by Chatterjee & Perkins (1966) that trichloroacetic acid extracts of vancomycin-inhibited cells contained a complex of precursor nucleotides with the antibiotic. However, no particular variation was observed in the results with different species or different trichloroacetic acid concentrations, so that we cannot explain why such complexes have been found more easily in plant-pathogenic corynebacteria than in Gram-positive cocci (Chatterjee & Perkins, 1966; Sinha & Neuhaus, 1968).

The uptake of vancomycin or iodinated vancomycin by cells of *M. lysodeikticus* was not greatly affected by the presence of 10mM-Mg^{2+}. Similarly Sinha & Neuhaus (1968) found that in 1ml of solution 0.75mg of cell walls (*M. lysodeikticus*) would adsorb about 950μg of vancomycin out of 1000μg. In the presence of 10mM-Mn^{2+} only 550μg was adsorbed, but when cell walls were added in excess this concentration of Mn^{2+} brought about only a 15% decrease in vancomycin adsorption. These results are in contrast with observations on *B. subtilis* W23 (Best & Durham, 1965) and also with our own results with iodinated vancomycin adsorbed on the purified mucopeptide of *B. licheniformis*. This mucopeptide is known to have very few un-cross-linked peptide chains that retain a D-alanyl-D-alanine *C*-terminus (Hughes, 1968). It therefore seems possible that the adsorption of vancomycin on the cell walls or mucopeptide of *B. subtilis* was relatively non-specific, whereas the binding to the cell walls of *M. lysodeikticus* may have involved considerable complex-formation with D-alanyl-D-alanine terminal sequences (Perkins, 1969). Provided that all non-specifically bound antibiotic could be preferentially removed, it should be possible to calculate the number of specific binding sites in a preparation of cell walls or membranes. So far we have not succeeded in this quest.

The presence in the membrane fraction of a small proportion of the vancomycin adsorbed by whole cells of both *M. lysodeikticus* and *B. subtilis* may well be an important indication of the action of the antibiotic. This portion could conceivably be attached to the disaccharide–peptide moiety of the lipid intermediate that plays a part in mucopeptide biosynthesis (Anderson *et al.* 1965, 1967; Struve, Sinha & Neuhaus, 1966; Higashi, Strominger & Sweeley, 1967). It is equally possible, of course, that attachment to the cell wall on D-alanyl-D-alanine termini may prevent further growth (Best & Durham, 1965; Perkins, 1969). The phenomenon of transfer of vancomycin from the

cell walls to the membrane fraction during prolonged incubation is difficult to understand. Although this could have been due to lysis of the cell walls, it is perhaps surprising that material released in this way should have attached itself to the protoplast membranes rather than have appeared in the sucrose supernatant, which after all contains a cell-wall digest produced by lysozyme. Insofar as it was possible to prepare a protoplast membrane fraction soon after the addition of vancomycin (because of the period required for digestion by lysozyme), such membranes contained 8% of the adsorbed vancomycin, corresponding to 1.6μg of antibiotic/mg dry wt. of cells. Thus, although the major part was then in the cell walls, a significant quantity was in the membranes. The antibiotic action of vancomycin could be exerted at either of these sites. At present we have not established whether the vancomycin that transfers to the membrane fraction carries with it a portion of mucopeptide with a D-alanyl-D-alanine terminus, though this seems possible.

Whole cells incubated with iodinated vancomycin even for a short period and then fractionated contained more antibiotic in the membrane fraction than did preformed protoplasts treated in the same way. It therefore seems possible that even in cells incubated for a short time with vancomycin some of the antibiotic found in the membrane fraction may have become fixed there because of a prior attachment to the cell walls.

We thank Mr J. B. Cole for technical assistance and Dr R. C. Hughes and Dr T. Diaz-Mauriño for purified mucopeptide samples.

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