The Specificity of Combination between Ristocetins and Peptides Related to Bacterial Cell Wall Mucopeptide Precursors

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The affinity of ristocetin B for analogues of the C-terminal tripeptide sequence of bacterial cell wall mucopeptide precursors resembles that of vancomycin. Complex-formation requires a D-configuration in the two amino acid residues of the C-terminal dipeptide, an L-configuration is preferred in the preceding amino acid residue and positive charges on the peptide molecule decrease its affinity. The specificity of ristocetin B, however, differs from that of vancomycin in the requirements for the size of the side chains on the C-terminal dipeptide. These differences may explain the observed differences in antibiotic behaviour of vancomycin and ristocetin with particular micro-organisms. The optical rotatory dispersion and u.v.-absorption characteristics of the ristocetins are very different from those of vancomycin but nearly identical with those of ristomycin A. Aglycones prepared from ristomycin A were antibiotically active and also combined with a specific peptide.

The ristocetins are antibiotics isolated from culture media of *Nocardia lurida* N.R.R.L. 2430 (Grundy *et al.* 1957), and whose mechanism of action is probably identical with that of vancomycin (Reynolds, 1966). Perkins (1969) added further support to this idea by showing that ristocetins A and B, like vancomycin, formed specific complexes with mucopeptide precursors or simple alanine peptides. All these peptides terminated in D-Ala-D-Ala. The purpose of the work described in this paper is to examine the specificity of ristocetin B for peptides in which alterations to the side chains of these and other residues have been introduced. A comparison between the specificities of ristocetin B and vancomycin (Nieto & Perkins, 1971b) is used in an attempt to explain differences in the inhibition pattern of both antibiotics, such as the ones found by Sinha & Neuhaus (1968).

MATERIALS AND METHODS

Chemicals. The preparation of all the peptides utilized in this work has been previously described (Nieto & Perkins, 1971b). Ristocetins A and B were kindly given by Abbott Laboratories, N. Chicago, Ill., U.S.A., and ristomycin A by Prof. G. F. Gause. The specific extinction of ristocetin B, corrected for loss of weight (11%) and ash (0.4%), was $E_{1\text{cm}}^{1\%} = 49.0 \pm 1$ at 280 nm.

Ristomycin aglycone. This was prepared from ristomycin A by methanolysis in 1M-HCl (reflux, 1h) as described by Lomakina, Muravyeva & Puskas (1968b), except that it was precipitated from solution at pH 9 by the addition of 20 volumes of acetone. The product was washed with acetone and purified by silica-gel t.l.c. in *n*-

butanol-acetic acid-water (3:1:1, by vol). The diazo-sulphanilic acid reagent (Ames & Mitchell, 1952) revealed two bands (R_F 0.05 and 0.42 respectively), neither of which contained sugars.

Association constants. Binding of peptides was measured by u.v. difference spectroscopy and association constants were deduced from Scatchard plots of the binding results as described by Nieto & Perkins (1971a). A very dilute solution of the antibiotic (35.4 µg/ml) in 0.02M-sodium citrate buffer, pH 5.1, was placed in silica fused tandem cells of 2 cm light-path and the difference in u.v. absorption developing at 243 nm upon addition of peptide was observed. The settings of the Unicam SP. 700 spectrophotometer were: resolution 3, damping selector 2, slit width 1.0-1.1 mm; the temperature was $26 \pm 1^\circ\text{C}$.

Optical rotatory dispersion was measured in a Polar-matic 62 spectropolarimeter (Bellingham & Stanley, London N.15, U.K.) as described by Nieto & Perkins (1971a).

RESULTS

To facilitate the description of the results the positions of amino acid residues within sequences are numbered in the order: H-3-2-1-OH. The sequence L-Lys-D-Ala-D-Ala is used as a standard for comparison of the effect of alterations in amino acid residues on the affinity for antibiotic. The molecular weight chosen for ristocetin, based on peptide titrations similar to those reported for vancomycin (Nieto & Perkins, 1971a), was 1800, for which value a Scatchard plot gave 0.98 combining sites per mol.

Specificity characteristics of ristocetin B. Table 1 compares the association constants (K_A) and free

Table 1. Association constants and free enthalpy changes for the combination of ristocetin B with peptides

The experiments were carried out as indicated in the Materials and Methods section. The corresponding values for vancomycin (Nieto & Perkins, 1971b) are included for comparison. The temperature was $26^\circ \pm 1^\circ\text{C}$. $\alpha\text{-Ac-L-Lys-D-Ala-D-Ala-NH}_2$ and $\text{Ac}_2\text{-L-Lys-D-Ala-Gly-NHOH}$ did not combine with either antibiotic. Abbreviations: Aib, α -aminoisobutyric acid; MurNAc, *N*-acetylmuramic acid; Hsr, homoserine.

Peptide	Ristocetin B		Vancomycin	
	$K_A(\text{l}\cdot\text{mol}^{-1})$	$\Delta G(\text{cal/mol})$	$K_A(\text{l}\cdot\text{mol}^{-1})$	$\Delta G(\text{cal/mol})$
Changes in residue 1				
1. $\text{Ac}_2\text{-L-Lys-D-Ala-D-Ala}$	5.9×10^5	-7850	1.5×10^6	-8400
2. $\text{Ac}_2\text{-L-Lys-D-Ala-Gly}$	2.2×10^4	-5900	1.3×10^5	-6950
3. $\text{Ac}_2\text{-L-Lys-D-Ala-D-Leu}$	6.1×10^5	-7860	9.2×10^3	-5390
4. $\text{Ac}_2\text{-L-Lys-D-Ala-D-Lys}$	1.0×10^5	-6800	1.4×10^4	-5620
5. $\text{Ac}_2\text{-L-Lys-D-Ala-L-Ala}$	No combination		No combination	
6. Ac-D-Ala-D-Ala	7.2×10^4	-6600	2.0×10^4	-5840
7. Ac-D-Ala-Gly	1.9×10^3	-4470	5.4×10^3	-5070
Changes in residue 2				
8. $\text{Ac}_2\text{-L-Lys-Gly-D-Ala}$	1.6×10^5	-7070	9.4×10^4	-6760
9. $\text{Ac}_2\text{-L-Lys-D-Leu-D-Ala}$	5.8×10^4	-6470	2.9×10^5	-7420
10. $\text{Ac}_2\text{-L-Lys-L-Ala-D-Ala}$	No combination		No combination	
11. $\text{Ac}_2\text{-L-Lys-Aib-Gly}$	No combination		No combination	
12. Ac-Gly-D-Ala	4.9×10^4	-6390	1.1×10^4	-5500
Changes in residue 3				
13. $\text{Ac-Gly-D-Ala-D-Ala}$	1.6×10^5	-7070	9.4×10^4	-6760
14. $\text{Ac-L-Ala-D-Ala-D-Ala}$	2.2×10^5	-7270	3.1×10^5	-7450
15. $\text{N-Ac-L-Tyr-D-Ala-D-Ala}$	2.9×10^5	-7430	1.9×10^5	-7180
16. $\text{Ac-D-Ala-D-Ala-D-Ala}$	1.3×10^5	-6960	5.0×10^4	-6380
Influence of free amino groups				
17. L-Lys-D-Ala-D-Ala	8.2×10^3	-5320	1.2×10^4	-5510
18. $\alpha\text{-Ac-L-Lys-D-Ala-D-Ala}$	1.9×10^5	-7200	4.7×10^5	-7700
Simultaneous changes in residues 1, 2 and 3				
19. Ac-L-Ala-Gly-Gly	2.5×10^3	-4620	4.9×10^3	-5200
20. $\text{Ac-Gly-Gly-Gly-Gly}$	8.0×10^2	-3950	1.5×10^3	-4300
21. $\text{Ac-L-Ala-D-Glu-Gly}$	6.8×10^2	-3850	4.8×10^5	-7720
22. <i>C. poinsettiae</i> dimer*	9.8×10^5	-8150	9.4×10^4	-6760

* The structure of this peptide (Leyh-Bouille *et al.* 1970) is as follows:

representing a mixture of molecules with and without the D-alanine residue shown in parentheses. For this peptide and ristocetin B, K_A was determined from only three experimental points, with an antibiotic concentration of 0.13 mg/ml.

enthalpy (Gibb's free energy) changes (ΔG) for the combination of the different synthetic peptides with ristocetin B and with vancomycin. For both antibiotics a free terminal carboxyl group was an absolute requirement for combination and so was an amino acid with a D-configuration or glycine in positions 1 and 2. An L-configuration was preferred for position 3 and when the amino group or groups in this residue were free, combination was very much weakened. The affinity of the standard peptide $\text{Ac}_2\text{-L-Lys-D-Ala-D-Ala}$ (peptide 1)* for

* Abbreviation: Ac, acetyl.

ristocetin B was somewhat less than for vancomycin. The main differences found in the specificity of ristocetin B as compared with that of vancomycin lay in the different size requirements for D-configuration side chains in positions 1 and 2.

Variations at residue 1. When the side chain of this residue, while in D-configuration, was increased in size from a methyl group (D-alanine; peptide 1) to an isobutyl group (D-leucine; peptide 3), the resulting peptide combined relatively poorly with vancomycin but very well with ristocetin B. Enhanced affinity for ristocetin and weaker binding of

vancomycin were also observed with the disaccharide peptide dimer from *Corynebacterium poinsettiae* (peptide 22), which likewise has a very large side chain in residue 1. Conversely, changing the terminal residue to a glycine resulted in a larger decrease in affinity for ristocetin B than in affinity for vancomycin. Thus it seems that the steric restrictions for binding imposed at position 1 of the peptide by ristocetin are less exact than those imposed by vancomycin. Furthermore, for ristocetin the optimum size for side chains in the D-configuration may be bigger than a methyl group. However, the charge at position 1 seems to be more critical for ristocetin B than for vancomycin. The peptide Ac₂-L-Lys-D-Ala-D-Lys (peptide 4) with a positively charged side chain in residue 1, combined better than Ac₂-L-Lys-D-Ala-D-Leu (peptide 3) with vancomycin but less well with ristocetin B. Thus there is presumably an electrostatic restriction at position 1 in the case of ristocetin B that is absent in the case of vancomycin. This might be related to the presence of an extra amino group per molecule of ristocetin as compared with vancomycin (Lomakina, Muravyeva & Yurina, 1970a; Nieto & Perkins, 1971a).

Variations in the side chain of residue 2. A different situation was found in the case of this residue. Increasing the size of the D-side chain from a methyl group (peptide 1) to an isobutyl group (peptide 9) decreased the affinity for vancomycin fivefold and for ristocetin B tenfold. Conversely, decreasing the size of the side chain from a methyl to a hydrogen (peptides 1 and 8) produced a fairly large decrease in the affinity of the peptide for vancomycin but only a minor one in the case of ristocetin B. Thus ristocetin B seems to be sterically more restrictive than vancomycin at residue 2. Given the specificity

shown by ristocetin B at residues 1 and 2 it is not surprising that Ac-L-Ala-D-Glu-Gly (peptide 21), a peptide with high affinity for vancomycin, combined very poorly with ristocetin B. The low affinity may also have been attributable to a charge effect. The relative affinities of other peptides in which simultaneous alterations in positions 1, 2 and 3 have been introduced (Table 1) could also have been approximately predicted from the specificity shown at positions 1 and 2.

Interactions contributing to the observed free enthalpy changes. Table 2 shows the contributions to the free enthalpy of combination made by different portions of the peptide molecule. The values for the so-called partial ΔG have been obtained by comparing overall values for peptides differing only in one side chain, taking as reference the corresponding peptide with a hydrogen atom in the position concerned (a glycine residue). With ristocetin B, as with vancomycin, it is clear that the peptide 'backbone' contributes about half of the free enthalpy of the binding, the rest of the contribution being made by the side chains. For both antibiotics these contributions of the side chains are not additive and hence not independent of each other. The presence or absence of other side chains as well as the overall length of the peptide determines to a large extent the contribution made to the binding by a particular side chain. Nevertheless, marked differences were observed in the contribution to the free enthalpies of binding by side chains in positions 1 and 2, depending on whether vancomycin or ristocetin was used.

Perturbations causing the u.v. difference spectra. The u.v. difference spectrum induced upon combination of ristocetin B with the standard peptide Ac₂-L-Lys-D-Ala-D-Ala (Fig. 1) is made of a negative

Table 2. Contributions made by interaction with different peptide side chains to the free enthalpy of combination between specific peptides and ristocetin B or vancomycin

The values of partial ΔG were obtained as indicated in the text. The overall values of ΔG for the combination with Ac₂-L-Lys-D-Ala-D-Ala were -7850 cal/mol for ristocetin B and -8400 cal/mol for vancomycin.

Contribution to ΔG made by	Peptides compared (nos. refer to Table 1)	Value of partial ΔG (cal/mol) for combination with	
		Ristocetin B	Vancomycin
Peptide 'backbone'	20	-3950	-4300
D-Methyl, residue 1	1 & 2	-1950	-1450
	6 & 7	-2130	-770
D-Isobutyl, residue 1	2 & 3	-1960	+1560
D-Methyl, residue 2	1 & 8	-780	-1640
	6 & 12	-210	-340
D-Isobutyl, residue 2	1 & 9	+600	-660
D-Methyl, residue 3	13 & 16	+110	+380
L-Methyl, residue 3	13 & 14	-200	-720
	19 & 20	-670	-900
L-4-Acetamido- α -butyl, residue 3	1 & 13	-780	-1640

difference with a minimum at 287 nm and shoulders at about 279 and 294 nm. A large negative difference develops further into the u.v. To try to account for the perturbations giving rise to this difference spectrum, the difference spectra induced by peptides having side chains of different sizes or other characteristics were recorded (Figs. 1 to 5). A striking feature is the fact that a peptide devoid of side chains, Ac-Gly-Gly-Gly-Gly, is able to produce a difference spectrum qualitatively similar to that of the standard peptide (Figs. 1 and 2). Addition of side chains to this peptide backbone only intensifies the difference and causes the shoulders at 279 and 294 nm to develop. In the case of the peptide Ac₂-L-Lys-D-Leu-D-Ala (Fig. 1) these two negative shoulders are transformed into two positive maxima thus supporting the idea that perturbations in these regions originate in a direct interaction of the side chains with phenolic chromophores.

It is also noteworthy that similar side chains at different positions in the peptide can induce

perturbations resembling each other; thus the shoulder at about 279 nm is induced by L-alanine at position 3 (Fig. 3) or D-alanine in position 1 (Fig. 2). Since on structural grounds it is very difficult to imagine that these two side chains interact with the same phenolic group in ristocetin B, it seems probable that they interact in the same way with different phenolic groups having very similar absorption characteristics. In no case are the difference spectra quantitatively additive, again suggesting that the perturbations causing them are not independent, so that to some extent the spectra reflect an interaction with the peptide as a whole.

When the side chain at position 3 (Fig. 4) was varied comparatively few qualitative changes in the near-u.v. difference spectrum were produced, although the presence of a L-tyrosine residue eliminated the negative shoulder at 279 nm as well as decreasing the quantitative difference at the minimum at 287 nm. Free amino groups decreased the interaction, but altered the shape of the difference spectrum to a surprisingly small extent (Fig. 5). The explanation of this might be that any electrostatic repulsion leading to a decreased affinity took place with a group on the ristocetin not closely connected to the phenol residues.

Ristocetin A and ristomycin A. Very little is known about the chemistry of the ristocetins. However, a group of Russian workers has recently reported the results of a fairly detailed study on the structure of ristomycin A (Lomakina, Bogнар, Brazhnikova, Sztaricskai & Muravyeva, 1970b). This is an antibiotic isolated from *Proactinomyces fructiferi* (Gause, Kudrina, Ucholina & Gavrilina, 1963) and believed to have a similar chemical composition and mode of action to the ristocetins. A sample of purified ristomycin A was obtained from Professor G. F. Gause. Its ability to combine with Ac₂-L-Lys-D-Ala-D-Ala was tested by u.v. difference spectroscopy and optical rotatory dispersion and compared with that of ristocetin A. The optical rotatory dispersion of both antibiotics was identical within experimental error and only minor quantitative differences were evident when the complexes with peptide were compared (Fig. 6). The u.v. difference spectra developed by the addition of the same peptide were also very similar (Fig. 7). Since the sugar moieties of ristomycin A and ristocetin A seem to be identical (Lomakina *et al.* 1968b; Lomakina, Spiridonova, Bogнар, Puskas & Sztaricskai, 1968c) and since we can expect the optical rotatory behaviour in this near-u.v. region of the spectrum to depend mainly on the phenolic chromophores, it seems reasonable to conclude that both antibiotics are identical or nearly identical. Small differences in the spectra of their complexes might be due to unequal purity of the samples. On the other hand, the u.v. absorption,

Fig. 1. Comparative effect of methyl and isobutyl side chains at residues 1 and 2 on the u.v. difference spectrum. U.v. difference spectra of ristocetin B induced by complex-formation with: (—), Ac₂-L-Lys-D-Ala-D-Ala; (---), Ac₂-L-Lys-D-Ala-D-Leu; (....), Ac₂-L-Lys-D-Leu-D-Ala. The concentration of ristocetin B was 0.10 mg/ml in 0.02 M-sodium citrate buffer, pH 5.1, and a large excess of peptide was added to ensure that all the ristocetin B was in the complexed form. The temperature was 26 ± 1°C and the spectrophotometer settings were as follows: damping selector 2; resolution 3; scan speed 3; slit adjustment, automatic. No expanded scale was used.

Fig. 2. Effect of methyl side chains at residues 1 and 2 on the u.v. difference spectrum. U.v. difference spectra of ristocetin B were induced by complex-formation with: (—), Ac-D-Ala-D-Ala; (---), Ac-Gly-D-Ala; (-·-·-), Ac-D-Ala-Gly; (....), Ac-Gly-Gly-Gly-Gly. Experimental conditions were as in Fig. 1.

Fig. 3. Effect of the side chain of residue 3 on the u.v. difference spectrum. U.v. difference spectra of ristocetin B were induced by complex-formation with: (—), Ac-L-Ala-Gly-Gly; (....), Ac-Gly-Gly-Gly-Gly. Experimental conditions were as in Fig. 1.

Fig. 4. Effect of the side chain of residue 3 on the u.v. difference spectrum. U.v. difference spectra induced on ristocetin B by complex-formation with: (—), Ac₂-L-Lys-D-Ala-D-Ala; (---), N-Ac-L-Tyr-D-Ala-D-Ala; (-·-·-), Ac-Gly-D-Ala-D-Ala; (....), Ac-D-Ala-D-Ala-D-Ala. Experimental conditions were as in Fig. 1.

optical rotatory dispersion and u.v. difference spectra obtained on complex-formation of the ristocetins and vancomycin with peptide are quite different and one would expect the aglycones to be chemically different in some major respects, even if their basic structure is similar.

The aglycones of ristomycin A were prepared by methanolysis (Lomakina *et al.* 1968b). The products were separated by t.l.c. into two bands that gave a yellow colour with diazotized sulphanilic acid, one

barely separated from the origin (aglycone 1) and one with R_F 0.42 (aglycone 2). Samples of the eluted materials were hydrolysed and shown to be free of sugar residues. Both aglycone 1 and aglycone 2 developed a u.v. difference spectrum when Ac₂-L-Lys-D-Ala-D-Ala was added and a maximum difference was soon reached, beyond which further

Fig. 5. Effect of free amino groups on the u.v. difference spectrum. Difference spectra of ristocetin B were induced by complex-formation with: (—), $\text{Ac}_2\text{-L-Lys-D-Ala-D-Ala}$; (---), $\alpha\text{-Ac-L-Lys-D-Ala-D-Ala}$; (....), L-Lys-D-Ala-D-Ala . Experimental conditions were as in Fig. 1.

Fig. 6. Optical rotatory dispersion spectra of (—), ristocetin A and (---), its complex with $\text{Ac}_2\text{-L-Lys-D-Ala-D-Ala}$; (---), ristomycin A and (....), its complex with the above peptide. The experiments were carried out in H_2O at pH 7.0 and 25°C . $E_{280}^{1\text{cm}}$ was 1.366 for ristocetin A and its complex and 0.864 for ristomycin A. The spectra for ristocetin B and its complex with peptide were virtually identical with those of ristocetin A and its complex respectively. The settings of the spectropolarimeter were: slits 1 mm; scale 50 m° or 100 m° ; scan time 37 min with time-constant 2.

Fig. 7. U.v. difference spectra induced by $\text{Ac}_2\text{-L-Lys-D-Ala-D-Ala}$ on (—), ristocetin A ($E_{280}^{1\text{cm}} = 1.38$) and (....), ristomycin A ($E_{280}^{1\text{cm}} = 1.53$). Other experimental conditions were as in Fig. 1.

addition of peptide had no effect. The final difference spectra (Fig. 8) may be compared with that of ristomycin A (Fig. 7). The difference spectrum of aglycone 1 retained the minima at 286 nm and in the far u.v., but the shoulder at 294 nm had disappeared and was replaced by a maximum at the same wavelength. This resembles the difference spectrum of vancomycin induced by combination with the same peptide (Nieto & Perkins, 1971b). The difference spectrum of aglycone 2 was relatively featureless, although once more a large negative difference developed in the far u.v. By assuming that aglycone 1 had $E_{278\text{nm}}^{1\text{cm}}$ of 64 (Lomakina *et al.* 1968b), the difference spectra were used to calculate the equivalent weight of combination with the peptide (Nieto & Perkins, 1971a). The resulting value of 1300 agrees fairly well with that of 1500 calculated from the specific absorption of the *NN*-diDNP derivatives by the Russian authors.

The antibiotic action of the aglycones was tested against *Micrococcus lysodeikticus* by the hole-in-plate method (Perkins, 1969). Concentrations of $50\text{ }\mu\text{g/ml}$ (calculated from E_{278} as above) were placed in the wells and the zones of no growth were compared with that given by ristomycin A. The diameters were as follows: ristomycin A, 17 mm; aglycone 1, 16 mm; aglycone 2, 13.5 mm. Evidently both aglycones were antibiotics, as expected from their ability to complex with the specific peptide.

DISCUSSION

Complex-formation between ristocetin B and specific peptides. The general model for complex-formation with ristocetin would be the same as that for vancomycin (Nieto & Perkins, 1971b) except that the

Fig. 8. U.v. difference spectra induced by $\text{Ac}_2\text{-L-Lys-D-Ala-D-Ala}$ on aglycone 1 and aglycone 2 from ristomycin A. The concentrations of the aglycones were such that (1) $E_{280} = 1.26$ and (2) $E_{280} = 1.06$. The solvent and other conditions were as in Fig. 1.

'cleft' holding the peptide would be wider around residue 1 and narrower around residue 2 of the peptide, whereas the reverse is true for vancomycin. A similar contribution to ΔG is made by the peptide backbone for both antibiotics, but for ristocetin increasing the size of the side chain of residue 1 of the peptide from a methyl to an isobutyl group had no effect on the affinity, whereas combination with vancomycin was adversely affected. These facts support the idea that one of the main interactions contributing to the binding with either antibiotic is a polar bond (ionic or hydrogen bond) between the carboxyl group of the peptide and an amino or hydroxyl group of the antibiotic in a hydrophobic environment in the complex. This bond would be insulated from the solvent by the peptide side chains and the effectiveness of this insulation would depend on the length of the peptide and the size of the side chains in relation to the precise geometry of the antibiotic.

Aglycones of ristomycin A. Ristomycin A combined with $\text{Ac}_2\text{-L-Lys-D-Ala-D-Ala}$ in a way similar to that of ristocetin. When the sugar residues were removed chemically to yield aglycones, these products were antibiotically active and also combined with peptide. Molecular models of the aglycone that were constructed to fit the incomplete structural data of Lomakina *et al.* (1968a; 1970b) only showed that it was possible to build a 'phenolic cleft' having the correct size to accept residues 1 and 2 of the peptide as postulated by Nieto & Perkins (1971b). However, there was no obvious manner of fitting the peptide Ac-D-Ala-D-Ala into the cleft in a way that was consistent with the known specificity of complex-formation. Many hydrogen-bond interactions were possible but none

of them seemed to coincide with an optimum geometrical 'fit'. Hence, more details about the structure of the complexes will have to await confirmation of the structure of the aglycone and, perhaps, X-ray-crystallographic studies.

Interaction of ristocetin B with bacterial cell walls. In addition to binding nearly as well as vancomycin to D-Ala-D-Ala termini, ristocetin will bind much more strongly to the mucopeptide crossbridges of bacteria of the chemotypes I and IV defined by Ghuyssen (1968). *M. lysodeikticus* mucopeptide, on the other hand, contains D-Glu-Gly sequences and therefore should combine firmly with vancomycin but only very weakly with ristocetin B (Table 1). Sinha & Neuhaus (1968) reported reversal of the inhibition caused by vancomycin and ristocetin in a *M. lysodeikticus* cell-free system that synthesizes mucopeptide, by cell walls from the same micro-organism. However, reversal did not occur to the same extent for both antibiotics: under conditions in which it was virtually complete for vancomycin, *M. lysodeikticus* cell walls achieved only about 30% reversal of the inhibition by ristocetin. This may be accounted for by the very low affinity of ristocetin for peptides ending in D-Glu-Gly, when compared with their affinity for vancomycin. Under the conditions in which the biosynthetic assay was carried out *M. lysodeikticus* cell walls adsorbed both antibiotics from solution. However, the amount of cell walls required for half-maximal adsorption of 1.0mg of antibiotic from solution was 0.68mg for vancomycin and 4.88mg for ristocetin (Sinha & Neuhaus, 1968). This difference in affinity is completely consistent with the above explanation. One could predict on these grounds that cell walls from *C. poinsettiae*, for instance, would be more effective in the reversal of inhibition by ristocetin than by vancomycin and that *Staphylococcus aureus* cell walls would be equally good for both antibiotics. Sinha & Neuhaus (1968) also reported a 50% inhibition of mucopeptide synthesis in the *M. lysodeikticus* biosynthetic system by 7.5 $\mu\text{g/ml}$ of ristocetin compared with 20 $\mu\text{g/ml}$ of vancomycin. This could be explained if in the preformed mucopeptide chains assumed to be the acceptors for the addition of new disaccharide-pentapeptide units there were D-Glu-Gly chains present. These chains would bind some of the vancomycin, which would thus become unavailable for inhibitory action, whereas binding of ristocetin by these peptide chains would be very weak.

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