COMPOUNDS FORMED BETWEEN NUCLEOTIDES RELATED TO THE BIOSYNTHESIS OF BACTERIAL CELL WALL AND VANCOMYCIN

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Vancomycin causes sensitive bacteria to accumulate amino sugar nucleotides related to the mucopeptide of their cell walls (Jordan, 1961; Reynolds, 1961). Both vancomycin and ristocetin interfere with the utilization of lipid-phospho-disaccharide-pentapeptide for mucopeptide synthesis in cell-free preparations from Staphylococcus aureus and Micrococcus lysodeikticus (Anderson, Matsuhashi, Haskin & Strominger, 1965; Struve, Sinha & Neuhaus, 1966). We have found that in the presence of vancomycin certain plant pathogenic corynebacteria accumulate not only a UDP-nucleotide-peptide reminiscent of the Park compound 3 (Park, 1952) but also a compound of the same nucleotide with a molecule of vancomycin. Similar substances accumulate in S. aureus and M. lysodeikticus, and ristocetin can also be bound to nucleotide in Corynebacterium poinsettiae.

Experimental

Bacteria were grown in nutrient broth and glucose in shaken flasks at 30° (or 37° for S. aureus). Overnight cultures were diluted into 10 volumes of fresh medium and shaken vigorously for another 2-3 hr. The rapidly growing cells were harvested and resuspended in the same medium to a cell density of 1 to 2 mg. dry wt./ml. and shaken with chloramphenicol (50 µg./ml.) (not in experiments with S. aureus) for 15 min. and then with added vancomycin (25 or 50 µg./ml.) for 1.5 hr. After centrifuging the packed cells were extracted at 0° with 4 times their volume of 25% (w/v) trichloroacetic acid (5% trichloro-acetic acid, as often used, extracted very little amino sugar nucleotide from

C. poinsettiae). After a second extraction the combined extracts were purified on a column of Sephadex G25 (Pharmacia Ltd. Uppsala) (Rosenthal & Sharon, 1964). Material absorbing at 260 mμ was eluted soon after the void volume, and was shown to contain bound N-acetylamino sugar (Reissig, Strominger & Leloir, 1955). The substances were then chromatographed on washed Whatman No. 3MM paper in ethanol-M-ammonium acetate pH 7.5 (7.5:3 by vol.). Compounds detected on the paper by UV absorption were eluted and analysed, sometimes after further chromatography in iso-butyric acid-0.5 M ammonia (5:3 by vol.).

Identification of components. UDP was identified on chromatograms after brief acid hydrolysis and was estimated by its absorption at 260 mμ. Samples of nucleotide hydrolysed in 6N HCl at 105° overnight were analysed on a Technicon automatic amino acid analyser. Homoserine had first to be converted from its lactone to the free amino acid by bringing the sample to pH 10 with $Ba(OH)_2$ solution and heating at 100° for 5 min. Components were also recognized after paper electrophoresis in 0.25 M formic acid, when they separated in the following order:- aspartic acid, glutamic acid, muramic acid, homoserine, alanine, glycine, glucosamine, ornithine (or lysine) and homoserine lactone.

Components found in acid hydrolysates of vancomycin, and used here for indications of its presence, were glucose (identified by paper chromatography and by the action of glucose oxidase ("Glucostat", Worthington Biochemical Corp.) aspartic acid and N-methylleucine (previously reported by Johnson, 1962, quoted by Lightbown, 1964). N-methylleucine was identified by paper chromatography, particularly in tert-butanol-4.25 N ammonia (4:1 by vol.), by conversion to its DNP-derivative, and by the characteristic pink colour given on chromatograms sprayed with 3% p-nitrobenzoyl chloride in pyridine (Plattner & Nager, 1948). It was measured quantitatively by elution of the colours given with ninhydrin (15 min. 100°) on papers run in tert-butanol-ammonia, and measurement at 570 mμ.

Vancomycin was given by Eli Lilly Ltd., ristocetin (Spontin) by Abbott Laboratories, N. Chicago, and N-methylleucine and its DNP-derivative by

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Results

Rapidly growing cultures of C. poinsettiae were treated with vancomycin and the accumulated amino sugar nucleotides were examined on paper chromatograms. There were two UV absorbing bands containing amino sugar and amino acids. The faster moving one ("fast nucleotide") was found to contain UDP, N-acetylmuramic acid, alanine, glutamic acid, glycine and homoserine in a molar ratio of 1:1:2:1:1:1, but no ornithine or other diamino acid (Chatterjee & Perkins, 1966). The rest of the material remained on the origin, and represented about 25-30% of the total amino sugar nucleotide in the original extract. This "slow nucleotide" was found to contain the same components as the fast one, but in addition glucose, aspartic acid and N-methylleucine were present. Since these components are known to be present in vancomycin (Johnson, 1962) it seemed probable that the antibiotic was present in the "slow nucleotide". The results of quantitative analysis given in Table 1 suggest that there is one mole of vancomycin (of molecular weight 1560, Johnson, 1962) for each mole of amino sugar nucleotide. Glycerol and fatty acids were not detected in the hydrolysates.

Table 1. Comparison of vancomycin, "fast nucleotide" and "slow nucleotide"
(Molar ratios are given relative to 1 mole of N-acetylmuramic acid)

	UDP	N-methyl leucine	Aspartic acid	Glutamic acid	Homo-serine	Alanine	Lysine or Ornithine	Glycine
Vancomycin	NIL	1.00*	0.99					
C. poinsettiae								
fast nucleotide	1.10	NIL	NIL	0.98	1.10	1.98	NIL	0.96
slow nucleotide	1.18	0.82	0.76	1.22	0.88	1.88	NIL	0.92
M. lysodeikticus								
fast nucleotide	1.04	NIL	NIL	1.10	NIL	3.20	1.11	NIL
slow nucleotide**	1.20	0.78	0.91	1.13	NIL	3.16	0.98	0.72

* Molar proportion taken as unity; there was no N-acetylhexosamine.

** In addition glucosamine was found (molar proportion 1.70).

We attempted to find out where vancomycin was linked to the nucleotide, after excluding the possibility of an ionic bond. "Slow nucleotide" was eluted by a gradient of LiCl from a column of DEAE-cellulose as a single peak containing both mucopeptide and vancomycin components. On paper electrophoresis at pH 3.5 the "slow nucleotide" moved towards the anode (not quite so far as the "fast nucleotide") whereas free vancomycin was cationic. After electrophoresis a sample of "slow nucleotide" eluted from the paper still contained the key components, glucose, aspartic acid and N-methylleucine, indicative of the presence of vancomycin. Best & Graham (1965) showed that vancomycin was adsorbed by whole cells and isolated cell walls of B. subtilis, and that this adsorption could be largely reversed by the presence of Mg^{++} or Ca^{++} (1.6mM). Such a linkage was not likely in the "slow nucleotide" since on chromatograms run in ethanol-(ammonium acetate 1M, magnesium acetate 0.3M) (7.5:3, by vol.) the vancomycin remained attached to the amino sugar nucleotide.

Both "slow nucleotide" and vancomycin were heated in 0.01N HCl for 5 min. at 100° and the products were subjected to paper electrophoresis at pH 4.6. UDP and glucose were set free from the nucleotide, and the rest of the vancomycin molecule remained attached to the N-acetylmuramylpeptide. Johnson (1962) reported that vancomycin very readily lost its glucose on heating in acid.

"Slow nucleotide" from other species. When similar experiments were carried out with S. aureus, M. lysodeikticus, Corynebacterium tritici, Corynebacterium flaccumfaciens and Corynebacterium insidiosum similar "slow nucleotides" containing vancomycin accumulated. The proportion of amino sugar nucleotide found as "slow nucleotide" was less in the cocci than in corynebacteria.

"Slow nucleotide" from ristocetin. Preliminary experiments have shown that cultures of C. poinsettiae incubated with ristocetin (20 µg./ml.) also accumulate a small proportion of a nucleotide containing ristocetin.

Addition of vancomycin to "fast nucleotide". When vancomycin was incubated with the "fast nucleotide" from C. poinsettiae, no "slow nucleotide" was

formed. If, however, a broken cell preparation of log phase cells of the same organism was added to the mixture, then as much as 60% of the "fast nucleotide" could become linked to vancomycin (Table 2). Park nucleotide isolated from S. aureus H was also linked to vancomycin by the broken cell preparation from C. poinsettiae.

Table 2. Enzymatic conversion of "fast nucleotide" to "slow nucleotide" by cell-free extracts

Log phase cells of C. poinsettiae were suspended in buffer containing Tris-HCl, 0.05M, pH 7.5; $MgCl_2$, 0.02M, and mercaptoethanol, 0.001M. The cell suspension (15 mg./ml.) was shaken for 2 min. with glass beads in a cooled Braun homogeniser. The supernate (40,000 r.p.m. Spinco, 2 hr.) containing 6-8 mg. of protein per ml. was used as the enzyme extract. Preliminary experiments showed that the bulk of the enzyme activity was in this supernatant fraction.

The complete incubation mixture contained in a total volume of 1.6 ml., nucleotide, 0.8 mg. of vancomycin (in Tris-HCl pH 7.5) and 0.5 ml. of enzyme extract. Other additions or omissions were as shown. The incubation was for 1 hour at 37°. The mixture was then treated with 25% trichloroacetic acid and nucleotides were isolated as before.

Source of "fast nucleotide"	Additions or omissions	"Slow nucleotide" formed	Amounts of "slow nucleotide" components found (μ moles)		
			N-acetyl hexosamine	N-methyl leucine	Aspartic acid
C. poinsettiae					
normal "fast nucleotide", 0.18μ mole	complete	+	0.118	0.101	0.075
	+ATP 0.2μ mole	+	0.120	0.112	0.081
	- vancomycin	-			
	- nucleotide	-			
cycloserine* nucleotide, 0.22μ mole	complete	-			
S. aureus H, 0.3μ mole	complete	+	0.124	0.148	0.108
	- vancomycin	-			
	- nucleotide	-			

* Cells inhibited with cycloserine (80 μg./ml.) yielded an amino sugar nucleotide similar to the "fast nucleotide" described, but lacking the alanine residues.

The effect of the vancomycin nucleotide compounds on synthesis of mucopeptide in cell-free systems is being studied.

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