Effects of Penicillin and Glycine on Cell Wall Glycopeptides of the Two Varieties of *Vibrio fetus*¹

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Actively growing strains of *Vibrio fetus* venerealis and *V. fetus* intestinalis, none of which produced penicillinase, were treated with inhibitory levels of penicillin or glycine, primarily to gain insight into the differential sensitivities of the two varieties to both of these compounds. Treatments induced the accumulation of uridine nucleotide glycopeptide precursors which contained amino sugars and amino acids in various molar ratios. Penicillin-induced nucleotides all contained muramic acid and sometimes glucosamine; they generally contained alanine, glutamic acid, diaminopimelic acid, and glycine. Approximately equimolar ratios of these components were observed in some compounds, but ratios varied considerably in others. Glycine-induced nucleotides contained muramic acid and, in some instances, glucosamine. Amino acids were detected only infrequently and usually in low molar ratios. The data suggest that penicillinase production, differences in the chemical composition of glycopeptide, and variations in modes of action of penicillin and glycine cannot individually account for the differential sensitivities of venereal and intestinal strains of *V. fetus* to these substances.

Two varieties of *Vibrio fetus* are recognized currently, on the basis of divergent ecologic and pathogenic properties among strains of the organism (1, 9, 11, 15, 19), which coincide with variations in certain physiologic characteristics (4, 6, 13). *V. fetus* intestinalis is more resistant to penicillin (16) and can tolerate higher levels of glycine (4, 13) than *V. fetus* venerealis. Because these properties imply differences in cell wall structure, the following experiments were designed to determine whether differential sensitivities to penicillin or glycine were due to penicillinase production, qualitative differences in glycopeptide structure, or, conceivably, variations in the mode of action of these substances, as reflected by the composition and relative proportions of nucleotide precursors which accumulated in their presence.

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Materials and Methods

*Vibrio strains.* Two strains each of *V. fetus* venerealis (UM and 25,170) and *V. fetus* intestinalis (23 and 65) were used. The source and key biochemical characteristics of each strain were tabulated (Table 1).

Strains were kept lyophilized until used, at which time they were subcultured weekly in semisolid medium. Strains were examined routinely for colonial dissociation (3), and only lines of cells in the smooth phase were used in cultural studies.

*Cultural methods.* A medium simulating Cystine Heart Agar (Difco) was prepared by dissolving 25 g of Beef Heart for Infusions (Difco), 20 g of Proteose Peptone (Difco), 4 g of dextrose, 5 g of NaCl, 25 mg of L-cysteine, 70 mg of Na2SO4, 0.3 mg of sodium thioglycollate, and 10 g of Trypticase (BBL) in 1 liter of distilled water, adjusting the pH to 7.2, and autoclaving. Solid and semisolid forms of this medium were prepared by adding 20 and 1.6 g of agar, respectively, per liter. A 10% blood-agar medium was prepared with defibrinated bovine blood.

Seed cultures were prepared by inoculating 1 ml of 24-hr semisolid culture into Blake bottles containing 100 ml of solid medium. The inoculum was overlaid with 5 ml of broth and incubated for 24 hr at 37 C in an atmosphere of 5% O2, 10% CO2, and 85% N2. Growth from four bottles was then transferred to 300
ml of broth in 2-liter Erlenmeyer flasks, which were incubated at 37 C in a rotary shaker (model G-26, New Brunswick Scientific Co., New Brunswick, N.J.) at 120 oscillations/min. A gas mixture composed of 2.5% O2, 10% CO2, and 87.5% N2 was passed through the flasks at the rate of 0.6 ft3/hr. After approximately 16 hr, the cells in each flask were separated aseptically by centrifugation at 4 C for 20 min at 12,000 X g, re suspended in two fresh flasks containing 300 ml of medium, and incubated for 4 hr. Penicillin or glycine, in concentrations judged to represent minimal inhibitory levels, were then added to each flask. In general, two or three flasks were inoculated with each compound, and an equal number were retained as controls. Incubation was resumed for 2 hr, after which the cells were collected by centrifugation and washed three times with cold, distilled water prior to extraction of nucleotides. At least three runs were performed with each of the four vibrio strains and, in order to have sufficient quantities for chemical analyses, cells treated in each respective fashion were pooled prior to extraction with trichloroacetic acid.

Extraction and separation of nucleotides. The methods of Strominger and co-workers (20, 21, 24) were followed in all essentials for extraction of cell sediments with 5% trichloroacetic acid, recovery of nucleotides by charcoal absorption, and separation by two-dimensional paper chromatography in isobutyric acid and 0.5 N NH4OH (5:3) and in ethyl alcohol and 1 N ammonium acetate (pH 7; 7:5:3), respectively. Nucleotide bases were characterized by their absorption spectra (21).

Chemical analyses of nucleotides. Nucleotides were hydrolyzed in 6 N HCl in vacuum-sealed ampoules for 6 hr at 100 C. The solution was dried, and traces of HCl were removed by dissolving the dried solution in water and evaporating it three times. The final product was dissolved in 1 ml of water. Amino acids and amino sugars were determined by ion-exchange chromatography for 21 hr in an amino acid analyzer (Technicon Co., Inc., Chauncey, N.Y.) by the use of a 140- by 0.6-cm column with a gradient elution system composed of three citrate buffers (pH 2.9, 0.25 m; pH 3.8, 0.25 m; and pH 5.0, 0.85 m). Total phosphorus content of the hydrolysates was determined by the procedure of Fiske and Subbarow (5).

Assays for penicillinase. The method of Perret (14) for iodometric assay was followed in its essentials. A known penicillinase-producing strain of Staphylococcus aureus was used as a control.

Antibiotic and glycine sensitivity tests. Tests were made on blood-agar containing two-fold ascending concentrations of potassium penicillin G, USP (Chas. Pfizer & Co., Inc., Brooklyn, N.Y.; 0.05 to 32 IU/ml) or glycine (0.5 to 12%, w/v), following the methods of Plastridge et al. (16). Minimal inhibitory levels were calculated by the method of Plastridge et al.

Growth curves. Determination of growth cycles was based on optical density measurements at 525 nm. Overnight growth from culture bottles was inoculated into 300 ml of broth medium in an Erlenmeyer flask, as described previously. Samples (5-ml) were removed from the flask at intervals of 2 to 4 hr, and optical density measurements were continued until no increase was observed. Growth curves were constructed by transposing absorbance values to equivalent cell concentrations, determined for each strain by plate counts.

RESULTS

Growth cycle of vibrio strains. Growth curves for the two venereal strains were virtually identical, as were those for the intestinal strains. Logarithmic growth began after 2 hr and lasted for approximately 4 and 10 hr in intestinal and venereal strains, respectively. The generation time for intestinal strains was approximately 1 hr; for venereal strains, 2 hr.

Penicillin and glycine sensitivity. Reactions within each variety of V. fetus appeared well defined, but marked differences existed between them (Table 1). In agreement with prior findings (4, 13, 16), V. fetus venerealis was much more sensitive to penicillin and markedly less tolerant to glycine than was V. fetus intestinalis.

Penicillinase production. No penicillinase was detected in any of the strains examined. Colonies growing on medium containing minimal inhibitory levels of penicillin were subcultured on medium containing higher levels of the antibiotic. The concentrations of penicillin were gradually increased to 5 IU for V. fetus venerealis and to 32 IU for V. fetus intestinalis. Mutants which grew on these concentrations of penicillin were selected and tested for penicillinase production. They were in every instance negative.

Chemical analysis of accumulated nucleotides. When the strains of V. fetus venerealis and intestinalis were treated with penicillin or glycine, a number of ultraviolet-absorbing spots were observed on chromatograms from extracts of the cell suspensions. None was observed from extracts of untreated cells from any of the strains. Analyses of hydrolysates of the nucleotides from penicillin-treated and glycine-treated cells are presented in Tables 2 and 3. Compounds are numbered in sequence from the point of origin, although it must be emphasized that, in the absence of standards, no association is suggested among compounds from different strains bearing the same number.

All of the uridine nucleotides contained muramic acid, and, in various combinations, glucosamine, alanine, glutamic acid, diaminopimelic acid, and glycine. Nucleotides with cytosine as the base contained neither amino sugars nor amino acids. All nucleotides contained approximately 2 moles of phosphorus per mole of base. Molar ratios of amino sugars and amino acids generally were unequal to those of the nucleotide base. In all but two instances, amino
sugar ratios were lower, a result consistent with the partial destruction of these substances during acid hydrolysis. Amino acid ratios were occasionally equal to or multiples of the base ratios, but generally below, although approaching, unity. Some compounds from penicillin-treated cells (UM-3; 23-2, 3; 65-1) contained glutamic acid in very low molar ratios (<0.2), and a few compounds from glycine-treated cells (23-3, 65-4, 5) contained glycine or alanine in similarly low ratios. Most compounds from penicillin-treated cells differed from those of glycine-treated cells in that they contained three to four amino acids, whereas the majority of those from glycine-treated cells contained only one or no amino acid.

### Discussion

The divergent effects of penicillin and glycine on the two varieties of *V. fetus* implied differences in cell wall structure, although the possibility had to be considered that penicillinase production influenced sensitivity to penicillin. Because none of the vibrio strains (even mutants selected for increased penicillin resistance) produced penicillinase, an effect of this enzyme may be excluded.

The composition of nucleotides accumulating in the four strains of *V. fetus* under the influence of penicillin (Table 2) suggests a structural similarity in the glycopeptide component of venereal and intestinal strains. The sequence of
The effect of inhibitory concentrations of glycine on venereal and intestinal varieties of *V. fetus*, as reflected by the composition of accumulated nucleotides (Table 3), is similar and appears essentially the same as that observed on *S. aureus* by Strominger and Birge (22). The composition of these products, most of which lack or contain only low molar proportions of amino acids, supports the contention (22) that glycine in excessive concentrations inhibits the addition of the initial alanine to uridine diphosphate-acetylmuramic acid. It also supports the contention that the normal position of glycine in the muramyl peptide of *V. fetus* is not, therefore, in direct linkage with the lactyl group of muramic acid, as is postulated for *M. lactium* (18).

The isolation of some nucleotide compounds, after penicillin and glycine treatment, which contained both muramic acid and glucosamine suggests that they are joined at this stage as the disaccharide. This is at variance with the structure of nucleotide precursors from other genera (10) and with the sequence of glycopeptide biosynthesis established for *S. aureus* and *M. lysodeikticus*, in which the formation of the disaccharide occurs later in the form of membrane-bound phospholipid intermediates (2). It is conceivable that, in some instances, these combinations represent unresolved mixtures of uridine diphosphate-glucosamine and uridine diphosphate-

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**Table 3. Analyses of nucleotides from glycine-treated cells**

<table>
<thead>
<tr>
<th>Type</th>
<th>Strain</th>
<th>Compound</th>
<th>Base</th>
<th>Amount extracted</th>
<th>Phosphorus</th>
<th>Glucosamine</th>
<th>Muramic acid</th>
<th>Glutamic acid</th>
<th>Glycine</th>
<th>Alanine</th>
<th>Diaminopimelic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>V. fetus venerealis</em></td>
<td>UM</td>
<td>U</td>
<td>0.16</td>
<td>1.80</td>
<td>0.62</td>
<td>1.49</td>
<td>1.01</td>
<td>0.28</td>
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<td></td>
<td>2 U</td>
<td>0.19</td>
<td>1.79</td>
<td>0.68</td>
<td>0</td>
<td>0.73</td>
<td>0</td>
<td></td>
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<td></td>
</tr>
<tr>
<td></td>
<td>3 U</td>
<td>0.12</td>
<td>1.79</td>
<td>0.64</td>
<td>0</td>
<td>0.35</td>
<td>0</td>
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<tr>
<td></td>
<td>25,170</td>
<td>U</td>
<td>0.16</td>
<td>1.76</td>
<td>0.66</td>
<td>0.48</td>
<td>1.31</td>
<td>0.74</td>
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<tr>
<td></td>
<td>2 C</td>
<td>0.19</td>
<td>1.79</td>
<td>0.73</td>
<td>0</td>
<td>0</td>
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<tr>
<td></td>
<td>3 C</td>
<td>0.18</td>
<td>1.80</td>
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<td>0</td>
<td>0</td>
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<tr>
<td><em>V. fetus intestinalis</em></td>
<td>23</td>
<td>U</td>
<td>0.45</td>
<td>1.81</td>
<td>0.83</td>
<td>0.19</td>
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<tr>
<td></td>
<td>2 U</td>
<td>0.44</td>
<td>1.82</td>
<td>0.71</td>
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<td>3 U</td>
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<td>1.82</td>
<td>0.78</td>
<td>0</td>
<td>0.12</td>
<td>0.91</td>
<td>0.53</td>
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<td>4 C</td>
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<tr>
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<td>65</td>
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<td>0.81</td>
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<td>0.93</td>
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<td></td>
<td>2 U</td>
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<td>0.51</td>
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<td>3 C</td>
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<td>1.86</td>
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<td>0</td>
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<tr>
<td></td>
<td>4 U</td>
<td>0.50</td>
<td>1.86</td>
<td>0.96</td>
<td>0.73</td>
<td>0</td>
<td>0.23</td>
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<tr>
<td></td>
<td>5 U</td>
<td>0.56</td>
<td>1.85</td>
<td>1.02</td>
<td>0.68</td>
<td>0.10</td>
<td>0.13</td>
<td></td>
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</table>

* Results are presented as moles per mole of base.
* Uridine and cytosine.
* Expressed as micromoles per 0.5 g of cells (wet wt).

Amino acids in the muramyl peptide of *V. fetus* was not established, but it may be inferred tentatively from these data and from those of other genera (7, 10, 12, 18) to be alanine-glutamic acid-diaminopimelic acid-alanine, with glycine possibly attached to glutamic acid, as in *Micrococcus lysodeikticus*, either interposed between glutamic acid and diaminopimelic acid (7), or linked in side-chain fashion to the ε-carboxyl group (12). The existence of glycine in muramyl peptide nucleotides is unusual, but it has been established in *Microbacterium lacticum* (18). Its presence in a majority of the penicillin-induced nucleotide compounds is strong evidence in favor of its location in the muramyl peptide of *V. fetus*. The reason for the generally low molar ratios of amino acids, and particularly the very low ratios of glutamic acid which occurred in some compounds from strains UM, 23, and 65, is not known. Some of these may represent the resultant composition of mixtures of nucleotides unresolved by the chromatographic procedure. It is, however, possible that nucleotide precursors with unequal molar ratios occur naturally and accumulate as a result of penicillin treatment. Young (25), in studies of cell wall autolyses of *Bacillus subtilis*, characterized peptides with unequal molar ratios of diaminopimelic acid, glutamic acid, and alanine and suggested that the concentration of such products might increase through penicillin treatment.
muramyl peptide, although the molar ratios of base, phosphorus, and amino sugars do not support this possibility. The resolution of this question will not be possible without more refined chromatographic separations of the vibrio nucleotide compounds and corroborative data on the identity of the products in question.

Differential sensitivities to penicillin and glycine in closely related species may be related to quantitative variations in glycopeptide content or in sites sensitive to the effects of either substance. It is equally possible that qualitative or quantitative differences in cell wall components overlying the glycopeptide alter the accessibility of or affect the activity of penicillin and glycine upon the glycopeptide substrate. The studies of Hugo and Stretton (8), wherein increased lipid content of cells within any of several bacterial species was associated directly with penicillin resistance, point in this direction and also offer a possible explanation for the generally greater resistance to penicillin of gram-negative species, in which much higher lipid contents occur naturally in the cell wall (17). To obtain an increased understanding of these factors, quantitative and qualitative comparisons are being conducted of glycopeptide and endotoxin (23) components from purified wall preparations of bovine vibrios in comparable stages of growth.

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LITERATURE CITED