Chromatographic Determination of the \( \alpha \)- and \( \beta \)-Amino Acid Residues in Pneumococcal C-Polysaccharide

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SUMMARY

The configurations of the amino acids in pneumococcal C-poly saccharide have been determined chromatographically after acid hydrolysis. The amino acids were coupled with an L-amino acid N-carboxyanhydride for formation of the corresponding L-D- and L-L-dipeptides; these derivatives were separated by ion exchange chromatography on an amino acid analyzer. The amount of racemization of alanine which occurred during the hydrolysis was determined by measurement of the amount of tritium incorporated into the amino acid after hydrolysis of the C-polysaccharide in tritiated HCl. After correction for the amount of \( \alpha \) or \( \beta \) isomer formed by racemization, it was concluded that the content of \( \alpha \)-alanine was variable (25 to 33\%) in the three preparations of pneumococcal C-polysaccharide studied. Each of the preparations contained glutamic acid (100\% \( \alpha \) isomer) and lysine (100\% \( \beta \) isomer).

EXPERIMENTAL PROCEDURE

Materials

The C-polysaccharide samples from a strain of rough pneumo cococcus, R 36A, were generously donated by Dr. T.-Y. Liu and Dr. E. C. Gotschlich. For Preparation 1, the cells were lysed slowly by autolysis as described by Goebel et al. (8). The cells were incubated for 3 days at 37\°C in 1 liter of 0.05 M sodium acetate, pH 7.5, in the presence of toluene and chloroform. The centrifugation, precipitation, and washing procedures were those described by Gotschlich and Liu (9).

Preparation 2 was obtained by rapid autolysis of the cells with 0.1% sodium deoxycholate for 15 min (4, 10). Lysis was terminated by heating at 100\°C for 10 min. The further purification of the C-polysaccharide has been described by Liu and Gotschlich (4).

Preparation 3 is the A fraction of C-polysaccharide purified by DEAE-Sephadex chromatography by Gotschlich and Liu (9). These authors pointed out that this particular fraction had a very high ratio of peptide subunits per residue of muramic acid.

The amount of C-polysaccharide that is isolated is approximately 10\% of the dry weight of the whole cells.\(^1\) The cell wall represents about 20\% of the dry weight of the cell (11); hence, the yield of C-polysaccharide from the cell wall is calculated to be about 50\%. Since the molar ratios of alanine, glutamic acid, and lysine were determined by the authors, the amount of racemization that takes place during the hydrolysis must be subtracted from the total amount of enantiomer present before the amount of \( \alpha \) or \( \beta \) isomer in the original material can be established. Treatment of the free amino acids under the conditions of acid hydrolysis may sometimes be used as a control for measurement of the racemization of amino acid residues during the hydrolysis. However, this control may not always be adequate because the amino acid residues in some sequences of a peptide may be subjected to increased racemization during the hydrolysis. In particular, with antibiotics (6) and cell wall peptides, in which unusual linkages are present, the possibility of an increased amount of racemization of certain residues must be considered.

In the present studies, the total amounts of \( \alpha \)- and \( \beta \)-amino acids in pneumococcal C-polysaccharide have been determined. The extent of racemization of alanine which occurred during the acid hydrolysis was measured by the amount of tritium incorporated into the amino acid after hydrolysis of the C-polysaccharide in tritiated HCl (7).

\(^1\) M. McCarty, personal communication.
and lysine in the C-polysaccharide are the same as those in the complete cell wall (12). The peptide portion of the C-polysaccharide is considered to be representative of the parent structure.

The amino acid N-carboxyanhydrides were obtained as previously described (5). Tritiated water (1 Ci per g) was purchased from New England Nuclear (Catalogue No. NET-0015).

**Methods**

**Hydrolysis of C-Polysaccharide**—For determination of the total amounts of D- and L-amino acids the samples were placed in acid-washed Pyrex tubes, either 10 x 75 or 18 x 150 mm; 0.4 to 2.0 ml of 6 N HCl were added and the tubes were sealed. With some samples the tubes were evacuated before they were sealed (7). The heating was carried out in an oven at 110° for 18 to 22 hours.

For determination of the amount of D- or L-amino acid formed by racemization during the acid hydrolysis, the samples were placed in a Pyrex tube, 10 x 75 mm; 200 μl of concentrated HCl and 200 μl of 3H2O were added. The tubes were not evacuated; they were sealed and heated as described above. The precautions used in the handling of 3HCl have been described previously (7).

**Isolation of Amino Acids**—The cell wall preparation (42 mg) was heated in 4 ml of 6 N HCl for 18 hours in an evacuated tube as described above. After removal of HCl on a rotary evaporator at 40–45°, the residue was dissolved in 0.2 M sodium citrate, pH 2.2 (14). The solution was passed through a Millipore filter (0.22 μ, No. GSWP01300) in a Swinny adapter (No. XX3001200) for removal of particulate material. An analytical run was carried out on the column, 0.9 x 62 cm, of an amino acid analyzer equipped with a flow cell scintillation counter (0.8% efficiency for tritium). The 0 to 0.1 absorbance scale was used with the 0.2- to 0.5-μmole levels needed for the determination of small amounts of the L-D-dipeptide present.

For determination of the amount of D or L isomer formed by racemization during the acid hydrolysis, the sample that had been heated in 6 N HCl was evaporated to dryness several times at 40–45° for removal of the excess tritium. The solution, in 0.20 M sodium citrate, pH 2.2, was applied to the ion exchange column of the amino acid analyzer equipped with a flow cell scintillation counter (0.8% efficiency for tritium). The 0 to 0.1 absorbance scale was used with the 0.2- to 0.5-μmole levels needed for this analysis (7). The amount of racemization formed by racemization during the hydrolysis was calculated from the specific radioactivity of the amino acid with the standard graph described previously (7).

**RESULTS**

**Analysis of Isolated Amino Acids**—Alanine (3.20 μmoles) and lysine (2.09 μmoles) were obtained from the hydrolysis of 42 mg of the C-polysaccharide (Preparation 1). The purity of each amino acid was verified by an analytical chromatogram. The ratio of alanine to glutamic acid of 1.6 for the isolated amino acids is lower than the corresponding ratio of 1.8 for the unfractionated hydrolysate; some alanine was probably lost during the isolation. The analysis also showed that some glycine and histidine (7 and 10% of alanine, respectively) as well as muramic acid were also present in the acid hydrolysate of the C-polysaccharide.

The configuration of each isolated amino acid was determined by chromatographic separation of the diastereoisomeric dipeptides as described above. Portions of the alanine and of the glutamic acid were coupled separately with L-leucine N-carboxyanhydride; lysine was coupled with L-glutamic acid N-carboxyanhydride. The relative amounts of the diastereoisomeric dipeptides were then determined by ion exchange chromatography on the amino acid analyzer (Table I, Preparation 1). These results show, for the first time, that the pneumococcal cell wall contains L-lysine and L-glutamic acid, as do other bacterial cell walls (11). About 28% of the alanine was found to be the D isomer in the hydrolysate of this cell wall preparation.

Since alanine is a good substrate for L-amino acid oxidase, the result for Preparation 1 was checked with the enzymic method. Treatment of the isolated alanine with the oxidase in 0.05 M sodium pyrophosphate, pH 8.3, for 16 hours at 35° led to a decrease of 29% of the alanine present as determined by amino acid analysis. A control with L-alanine treated under the same conditions resulted in the oxidation of 51% of the racemic amino acid. Thus, both experimental methods indicate the same amount of D-alanine in the hydrolysate of this preparation of C-polysaccharide.

**Analysis of Unfractionated Hydrolysates**—Other preparations of the C-polysaccharide were analyzed for D-alanine content. Since bacterial cell walls contain relatively few amino acids, the mixture of dipeptides prepared by derivatization of the total hydrolysate could be chromatographed without fractionation. With Preparation 2 (Table 1), 5.8 mg of C-polysaccharide, pre-
pared by lysis with detergent, was hydrolyzed in 0.4 ml of 6 N HCl for 22 hours; the tube for hydrolysis was not evacuated before it was sealed. The ratio of alanine to glutamic acid to lysine in the hydrolysate was 2.0:1.0:0.8. After removal of the HCl, the residue was dissolved in sodium borate, pH 10.2, and a portion of the hydrolysate was coupled with L-leucine N-carboxy- anhydride. An aliquot of the dipeptide solution was chromatographed on an amino acid analyzer (0 to 0.10 absorbance scale) by elution with 0.20 M sodium citrate, pH 4.25, at 52°C. Under these conditions L-Leu-β-Ala and L-Leu-L-Ala are separated from all other components of the mixture. The results indicate that this cell wall preparation contained about the same percentage of β-alanine as did Preparation 1. Not only is less material required when the hydrolysate is analyzed directly for β- and L-amino acid content, but the probability of preferential loss of one enantiomer is diminished.

An experiment with a third preparation of C-polysaccharide, which had been purified by chromatography on DEAE-Sephadex (9), was examined next. Preparation 3 (1.7 mg) was heated in 1 ml of 6 N HCl for 18 hours. The ratio of alanine to glutamic acid in the hydrolysate was 2.0:1.0. After removal of the HCl on a rotary evaporator, the complete hydrolysate was coupled with L-leucine N-carboxy anhydride. Chromatography of the dipeptide mixture was carried out as described above for Preparation 2. The results of the analysis for Preparation 3 show that the amount of β-alanine present is significantly higher than found with the two other preparations.

**Correction for Racemization during Acid Hydrolysis**—The amount of β- and L-alanine that were formed by racemization was determined by hydrolysis of 5.8 mg of the C-polysaccharide (Preparation 2) in 400 μl of 6 N HCl as described above. From the specific radioactivity of the alanine, it was calculated that 2.7% of the β and L isomers of alanine were formed during the hydrolysis. This is nearly 3 times the amount of racemization found for free L-alanine under the same conditions (7). The ratio of L- to β-alanine for Preparation 2 is close to 3:1. Assuming that inversion of the alanine residues does not take place during the hydrolysis, then the amount of β isomer formed by racemization of the L-alanine residues is 2.1% while the amount of β isomer formed by racemization of β-alanine residues is 0.7%. The net amount of β-alanine formed is 1.4%. Subtraction of this value from the total amount of β-alanine present indicates that nearly 1 of every 4 residues of alanine in pneumococcal C-polysaccharide in Preparations 1 and 2 is the β isomer. Assuming that about the same total amount of racemization has occurred with Preparation 3, then the net amount of β-alanine formed by racemization during acid hydrolysis is 0.9%. Subtraction of this value from the total amount of β-alanine indicates that for this preparation, 1 of every 3 alanine residues is the β isomer.

The small amounts of L-glutamic acid and of β-lysine found are most likely formed by racemization during the acid hydrolysis (7, 17). The amount of racemization of glutamic acid cannot be determined by the tritium method (7), and there were interfering radioactive compounds that eluted close to lysine on the short column of the amino acid analyzer.

**Discussion**

Ikewa and Snell (1) found that the amounts of β-alanine in the cell walls of a group of closely related bacteria ranged from 32 to 66%. These authors concluded that the same cell wall structure may not be common to all bacterial species. Recently, Osborn (18) has compared the structures of the peptidoglycans of several bacteria where the extent of cross-linking varies from one species to the next. The results of the present study indicate that there may be diversity with respect to the proportions of β- and L-alanine within the same species.

The results described here could be interpreted to indicate that in the tetrapeptides (β-Ala-Glu-Lys-Ala) attached to the muramic acid residues of the C-polysaccharide (12), the distribution of β- and L-alanine residues is variable for different preparations. An alternative explanation is that the preparations contain a mixture of muramyl tetrapeptides, muramyl tripeptides that lack the COOH-terminal β-alanine residue (19), and muramyl L-alanine (20).

The methods for the determination of amino acid configurations utilized in this communication should be generally useful for the chemical study of cell walls; the analyses can be carried out rapidly and minimal amounts of material are used.

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**Table I**

**Proportions of β- and L-amino acids in hydrolysates of pneumococcal C-polysaccharide**

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>β Isomer (%)</th>
<th>L Isomer (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysine</td>
<td>2.5</td>
<td>97.5</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>94.7</td>
<td>5.3</td>
</tr>
<tr>
<td>Alanine</td>
<td>28.4</td>
<td>71.6</td>
</tr>
<tr>
<td>Preparation 2</td>
<td>28.7</td>
<td>71.3</td>
</tr>
<tr>
<td>Alanine</td>
<td>33.5</td>
<td>66.5</td>
</tr>
</tbody>
</table>

*These values include the amounts of enantiomers formed by racemization during acid hydrolysis.*

1 The amino acids were isolated from a hydrolysate of 42 mg of C-polysaccharide. The C-polysaccharide was prepared from cells which were lysed by autolysis.

2 C-Poly saccharide, prepared from cells lysed with sodium deoxycholate, was used. The unfraccionated hydrolysate was analyzed.

3 A preparation of C-polysaccharide purified by DEAE-Sephadex chromatography was used. The unfraccionated hydrolysate was analyzed.
REFERENCES
Chromatographic Determination of the d- and l-Amino Acid Residues in Pneumococcal C-Polysaccharide

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