The Diaminohexose Component of a Polysaccharide
Isolated from Bacillus subtilis*

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During the study of the acid hydrolysate of a polysaccharide from Bacillus subtilis (3), the hexosamine fraction (4) was found to give an unidentified spot on paper chromatograms. Its color reactions were typical for an amino sugar, but it moved at a faster rate than glucosamine or galactosamine. Furthermore, degradation with ninhydrin (5) afforded a reducing product possessing a Rf higher than that of unsubstituted pentoses or deoxy-pentoses (6).

The present paper describes the isolation of the amino sugar and its identification as a diaminohexose.

METHODS

Analytical Procedures—Hexosamines were estimated by the Gardell modification (7) of the Elson and Morgan procedure. Reducing sugar tests were run by the method of Somogyi (8), Garde11 modification (7) of the Elson and Morgan procedure. Reducing sugars were detected with silver nitrate, with the use of a 10-minute heating time, and the color reagent of Nelson (9). Free amino groups were assayed by the ninhydrin method of Cocking and Yemm (10) and by the alkaline distillation method of Tracey (11). Glucosamine hydrochloride (c.p., Pfanstiehl) was used as a standard.

Paper Chromatography—Chromatograms were run descending for 18 hours at room temperature, with Whatman No. 1 filter paper. The following solvents were used routinely: n-butanol-ethanol-water 4:1:1 and n-butanol-acetic acid-water 25:6:25. Reducing sugars were detected with silver nitrate, with the method of Trevelyan et al. (12). Amino sugars were detected by the Elson and Morgan (14) or ninhydrin (0.2% solution in n-butanol saturated with water) reagents.

Paper Electrophoresis—The electrophoresis was carried out in borate buffer at pH 10, according to the method of Bourne et al. (15).

EXPERIMENTAL AND RESULTS

Preparation of Polysaccharide—Bacillus subtilis (ATCC 9945)* was grown on Sauton's medium (16) in Fernbach flasks containing 500 ml of medium each. After 3 days of growth at 36°, the cells were collected by centrifugation in a cooled Sharples centrifuge and washed with cold distilled water. The succeeding steps were carried out at a temperature of between 0 and 5°. Two volumes of 10% trichloroacetic acid were added to the packed cells, mixed well, and the thick suspension was left overnight. On the next day, it was centrifuged for 30 minutes at 12,000 X g, the supernatant solution was poured off, and the cells were extracted twice more with trichloroacetic acid. The residue was discarded and the combined highly viscous extracts were left standing for 1 to 2 days, and centrifuged again for 30 minutes at 12,000 X g, resulting in a clear greenish solution. This last step was found to be essential for obtaining clear extracts.

The solution was then extracted three times with equal volumes of ether to remove the trichloroacetic acid. Upon the addition of 3 volumes of 0.5% ethanol to the aqueous solution, a white voluminous precipitate separated. It was washed with aqueous alcohol, absolute alcohol, and ether, and dried, first in vacuum and finally in an oven at 90°. Ninety-five liters of medium yielded 600 g of cells, which gave 7.5 g of crude polysaccharide. This product was satisfactory for the preparation of the diamino-hexose reported in this paper. Purer preparations of the polysaccharide (3) were, however, used in the preliminary experiments.

Hydrolysis of Polysaccharide—The crude polysaccharide (7.8 g) was dissolved in 180 ml of 1 N sulfuric acid, and heated under reflux in a boiling water bath for 6 hours. After cooling, the dark brown solution was neutralized with BaCO₃ filtered through a layer of Celite and charcoal (Darco G-60), and the precipitate was washed with 400 ml of warm water. The excess of Ba ions was removed from the combined filtrates by careful addition of an equivalent amount of dilute sulfuric acid, followed by filtration. The clear, neutral hydrolysate was lyophilized, and gave 5.1 g of a powdered product containing 20% hexosamines.

Fractionation of Hydrolysate—Although the hydrolysate could be fractionated directly on an ion exchange column with high resolving power (7), this process would have involved the use of a very large column and of large eluent volumes. A preliminary fractionation was therefore carried out in order to remove the
neutral sugars and the bulk of amino sugars and amino acids from the hydrolysate, leaving a fraction enriched with the unknown compound henceforth to be named Compound A.

A 2.4 x 17 cm Dowex 50 column prepared according to Boas (4) was used. The lyophilized hydrolysate was dissolved in 80 ml of water and added to the column. The neutral sugars were eluted from the column by washing with 600 ml of water, and the bulk of hexosamines (about 60%) by the use of 250 ml of 0.5 N HCl. The column was then washed with 400 ml of 1 N HCl and a fraction composed mainly of n-glucosamine, d-galactosamine, and Compound A was obtained. It accounted for 30% of the hexosamine content of the hydrolysate, and contained the major part of the Compound A, present in traces only in the first two fractions. Further washing of the column with 2 N HCl yielded only traces of amino sugars, and mainly amino acids, of which alanine was identified chromatographically as the major component.

The 1 N HCl fraction was concentrated to dryness in vacuum at room temperature. The dry material was dissolved in 20 ml of 0.3 N HCl, and chromatographed on a 3.5 x 33 cm Dowex 50 column, according to the method of Gardell (7), fractions of 10 ml being collected. The peaks of glucosamine and galactosamine emerged at about 1.0 and 1.2 liters, respectively, but no attempt was made to isolate these compounds from the eluate. Compound A started to emerge at 3.4 liters, reaching a broad peak at about 3.7 liters. The pooled fractions (600 ml from 3.4 to 4.0 liters) were evaporated by distillation in vacuum. The resulting material consisted chiefly of the unidentified amino sugar, but still contained small amounts of impurities, apparently glucosamine and galactosamine, and traces of amino acids. It was, therefore, further purified by paper chromatography.

The material from the column peak was dissolved in 5 ml of water and chromatographed on eight sheets of Whatman No. 1 filter paper, which had been extensively washed with distilled water and with ethanol. The mixture n-butanol-ethanol-water 4:1:1 was used as solvent, and the chromatograms were run descending for 24 hours. Guide strips were cut from the dried chromatograms and sprayed with silver nitrate (12). The portions containing Compound A thus identified were cut from the chromatograms and eluted with 200 ml of water. The eluate was concentrated to a small volume, a few drops of 1 N HCl added, and the solution was filtered through a double layer of Celite and charcoal. The filtrate was evaporated under a stream of nitrogen and the last traces of free hydrochloric acid were removed by repeated addition of absolute ethanol and evaporation with nitrogen. After drying in vacuum, 112 mg of a solid mass, partially crystalline, were obtained. This material was recrystallized from a mixture of water, methanol, and acetone, yielding 71 mg of colorless prismatic needles.

Characterization of Compound A—Crystalline Compound A was homogenous as determined by paper chromatography in the two mixtures of solvents described above (the Rfvalues are given in Table I) as well as in the three following mixtures of solvents (the Rfvalues and Rfvalues respectively are given in parentheses): (a) n-butanol-pyridine-water 6:4:3 (1.30, 1.70; diffuse spot, tailing); (b) ethyl acetate-pyridine-water 2:1:2 (1.03, 1.38; elongated spot); (c) n-butanol-ethanol-water 5:1:4 (1.3, 1.8; elongated spot). It was also homogenous in the mixture n-propanol-1% ammonium hydroxide 7:3, with a Rf of 1.38. On paper electrophoresis in borate buffer no movement of Compound A could be observed.

Compounds A reacted with the silver nitrate, ninhydrin, and Elson and Morgan sprays. It gave, however, no reaction with the anilne phthalate reagent. The crystalline material was soluble in water, methanol, and ethanol. It melted at 216–219° (corrected), with decomposition starting at 200° and had a mal-taration from [α]D20 +115° (after 5 minutes) to +94° (23 hours) (ε 0.05 in water).

The analytical values obtained for Compound A are reported in Table II. They correspond to C6H12O6N4Cl. When assayed by various colorimetric methods, the optical densities increased linearly in the following ranges tested: for alkaline distillation of ammonia, from 225 to 450 μg; for the hexosamine test, from 26.6 to 106.7 μg; for the reducing sugars test, from 53 to 214 μg; for the ninhydrin test, from 5.3 to 26.6 μg.

The absorption spectrum of the Elson and Morgan color (7) was similar to the one of glucosamine hydrochloride. Measurement with a Cary recording spectrophotometer gave almost identical absorption curves with a peak at 525 μm, and a ratio of 0.9 between the optical densities at 540 μm and 510 μm and 1.25 between the optical densities at 550 μm and 510 μm. With the Boas modification of the Elson and Morgan test (4), a ratio of 1.47 was obtained between the optical densities at 540 and 510 μm for Compound A, whereas, under the same conditions, glucosamine hydrochloride gave a ratio of 1.32.

Periodate Oxidation Studies—When Compound A was oxidized

**Table I**

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Chromatographic solvent mixtures</th>
<th>Electrophoresis in borate buffer at pH 10</th>
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<tbody>
<tr>
<td></td>
<td>n-Butanol-ethanol-water (4:1:1)</td>
<td>Rglucose</td>
</tr>
<tr>
<td></td>
<td>n-Butanol-acetic acid-water (25:6:25)</td>
<td>Rglucose</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Nglucose</td>
</tr>
<tr>
<td>Compound A</td>
<td>1.4</td>
<td>1.8</td>
</tr>
<tr>
<td>Compound B</td>
<td>4.5</td>
<td>3.3</td>
</tr>
<tr>
<td>Compound C</td>
<td>0.55</td>
<td>0.65</td>
</tr>
<tr>
<td>Compound D</td>
<td>5.8</td>
<td>4.5</td>
</tr>
<tr>
<td>d-Gluosamine HCl</td>
<td>0.0</td>
<td>0.7*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.23</td>
</tr>
</tbody>
</table>

* Double spot; main spot used for measurement.

**Table II**

<table>
<thead>
<tr>
<th>Analysis</th>
<th>Calculated for C6H12O6N4Cl</th>
<th>Found</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>Carbon*</td>
<td>39.90</td>
<td>40.16; 39.99</td>
</tr>
<tr>
<td>Hydrogen*</td>
<td>7.12</td>
<td>7.05; 7.05</td>
</tr>
<tr>
<td>Nitrogen (Dumas)*</td>
<td>11.64</td>
<td>11.60</td>
</tr>
<tr>
<td>Chlorine*</td>
<td>14.73</td>
<td>14.82</td>
</tr>
<tr>
<td>C=CH₂ (Kuhn-Roth)*</td>
<td>12.5</td>
<td>10.5</td>
</tr>
<tr>
<td>Free amino N⁺</td>
<td>5.82</td>
<td>4.7</td>
</tr>
<tr>
<td>Hexosamine†</td>
<td>88</td>
<td></td>
</tr>
<tr>
<td>Reducing sugars†</td>
<td>84</td>
<td></td>
</tr>
<tr>
<td>Ninhydrin†</td>
<td>94</td>
<td></td>
</tr>
</tbody>
</table>

* These microanalyses were carried out by Dr. R. Ritter, Dasei, Switzerland.
† Estimated by alkaline distillation (11).
‡ Molar values using glucosamine hydrochloride as standard.
by periodate according to the method of O'Dea and Gibbons (17), no formaldehyde could be detected, even after 24 hours of incubation. The test for acetaldehyde was carried out with a modification of the method of Nicolet and Shinn (18). The p-hydroxydiphenyl reagent (19) was used for the colorimetric estimation of the acetaldehyde formed with L-rhamnose serving as a standard. When the test was carried out immediately after mixing the reagent, 0.23 mole of acetaldehyde per mole of Compound A was formed; the acetaldehyde yield increased to 0.4 mole after 5 hours of incubation in the dark at room temperature and decreased to 0.26 mole after 18 hours of incubation.

Formic acid also was produced upon oxidation by periodate. It was estimated quantitatively in the Warburg respirometer, by the method of Perlin (20), with an incubation temperature of 25°. The results indicate that 1.7 moles of formic acid were formed after 24 hours from 1 mole of Compound A. Low values were obtained in the oxidation of the two known compounds tested under identical conditions, i.e. 0.8 mole formic acid per mole of methyl α-D-glucopyranoside and 3.6 to 4.5 moles formic acid per mole of D-glucosamine hydrochloride.

Degradation with Ninyhydrin—Compound A was oxidized by ninhydrin in a capillary tube according to the method of Stoffyn and Jeanloz (5). The resulting product (Compound D), which was not isolated, moved very fast on paper chromatograms (Table I). Its movement was faster than that of the fast moving pentoses (ribose or deoxyribose), methylpentoses (e.g. rhamnose), or methyltetroses (obtained by periodate oxidation of methylpentoses). The product was reducing, but was negative to the aniline phthalate reagent. It exhibited considerable movement on paper electrophoresis (Table I).

Acid Treatment—Action of 4 N HCl on Compound A for 6 hours at 100° converted it almost completely to a Compound C, which had a migration on paper chromatograms similar to that of glucosamine or galactosamine. It could be separated distinctly from these hexosamines in n-propanol-1% ammonium hydroxide with a Rf glucosamine of 1.09. It reduced silver nitrate and reacted positively to the ninhydrin and Elson and Morgan sprays.

Acetylation of Amino Group—To a solution of 6.5 mg of Compound A in 0.7 ml of methanol, 7 mg of silver acetate were added, followed by one drop of acetic anhydride. The mixture was left overnight in the dark at room temperature. It was then heated to boiling, cooled, and filtered through a layer of Celite and charcoal, and the filter washed. The combined filtrates were evaporated to dryness under a stream of nitrogen. The residue was dissolved in a small amount of water, and the last traces of silver ions removed by filtering through a thin bed of Dowex 50. The filtrate was dried and recrystallized from a mixture of ethanol and ethyl acetate to give 6.8 mg of fine needles or plates, m.p., 262-264° (corrected) with decomposition. The crystalline derivative (Compound D) dissolved sparingly in water, but was soluble in ethanol. Its rotation at equilibrium was $\alpha^D_{E} +67^\circ$ (c 1.09 in water-ethanol 1:1).

$$C_{13}H_{26}O_{4}N_2$$
Calculated: C 48.77, H 7.37
Found: C 49.36, H 7.90

On paper chromatograms, it moved faster than Compound A but exhibited no movement in paper electrophoresis. It reduced the silver nitrate spray but up to 210 µg gave no color in the Morgan and Elson test (21).

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**Infrared Spectra**—The infrared spectra of Compounds A and B were determined on a Perkin-Elmer apparatus, model 21 with a NaCl optic, in a KBr microspellet. The spectrum of Compound A is reproduced in Fig. 1.

**DISCUSSION**

The experiments described above show that a polysaccharide from *Bacillus subtilis* contains an amino sugar different from D-glucosamine and D-galactosamine, the usual amino sugar components of complex polysaccharides. This compound comprises between 1 and 2% of the polysaccharide, or about 0.1 to 0.2% of the dry weight of the bacterial cells, and it has been obtained in a pure form and crystalline state.

The elemental analysis of Compound A is consistent with the formula $C_{n}H_{26}O_{4}N_2Cl$ or a multiple of it. A direct molecular weight determination could not be performed, because of low solubility, but the reducing properties, the quantitative Elson and Morgan and ninhydrin reactions, as well as the movement on paper chromatograms indicate a monosaccharide. The presence of an α-amino-aldehyde grouping can be deduced from the reducing properties and the positive Elson and Morgan and ninhydrin reactions, whereas the liberation of acetaldehyde by periodate oxidation allows the assignment of a methyl group, vicinal to a secondary alcohol group at the other end of the chain. The C-methyl Kuhn-Roth determination shows the presence of two methyl groups in Compound A. Elemental analysis, resistance to periodate oxidation, and examination of the infrared spectrum, indicate that this second methyl group is part of an acetyl group linked to an amine. Strong infrared absorption bands appear at 1660 cm⁻¹ (amide I), at 1520 cm⁻¹ (amide II), and at 3365 cm⁻¹ (secondary amide), whereas the absorption band at 1725-1749 cm⁻¹ characteristic of the O-acetyl carbonyl group is lacking (22). Since the acetyl group contains two carbons, the backbone of the sugar is made of six carbons, and Compound A is a hexose.

The various reactions reported previously (Elson and Morgan, 1960), by guest on September 8, 2017 http://www.jbc.org/ Downloaded from
The infrared spectrum showed absorption bands only at 1640 cm⁻¹ (amide I), 1540 cm⁻¹ (amide II), and 3355 cm⁻¹ (secondary amide), characteristic of the amide group (22). The assignment of the second nitrogen of Compound A to a primary amine linked to acetic acid is less evident. It is based on the elemental analysis, and on the lack of absorption characteristic for a secondary or tertiary amine in the infrared spectrum. The treatment with strong acid releases a product which moves much more slowly on paper chromatography and is consequently more polar (Compound C). This behavior is in agreement with the liberation of a primary amine by cleavage of the amide linkage.

The presence of an acetylamino grouping in Compound A after hydrolysis of the polysaccharide with 1 N sulfuric acid for 6 hours at 100° is quite surprising. However, it is possible that this stability is due to the distance from the aldehyde group and the presence of a free amino group at C-2. The most probable location of the acetylamino group is at C-4, the two other positions at C-5 and C-3 being unlikely for the following reasons. Compound A showed a mutarotation requiring the presence of a free hydroxyl group at C-4 or C-5. The linkage of an amino group at the latter position is excluded by the formation of acetaldehyde after periodate oxidation of Compound A. Elimination of C-3 as a possible location is based on the identity of the absorption curves of Compound A and d-glucosamine after Elson and Morgan reactions, since substitution at C-3 has been previously shown to shift markedly the maximum of the curve (23, 24). The liberation of 1.7 moles of formic acid after periodate oxidation is also in better agreement with a substitution at C-4 than at C-3. An additional argument for the location of the acetylamino group at C-4 is the failure of Compound B to give a positive Morgan and Elson test. This lack of color formation has been previously linked to the inability of N-acetylamino sugars substituted at C-4 to produce the necessary furanose form of the chromogen (25, 26). It is possible to reconcile the presence of an acetylamino grouping at C-4 with periodate oxidative cleaving between C-4 and C-5 to give acetaldehyde, since it has already been reported that acetylation of an amino group vicinal to a free hydroxyl group does not inhibit oxidative cleaving by periodate (27, 28). However, it distinctly slowed down the reaction and the concomitant destruction of the liberated acetaldehyde by the excess of periodate reagent would explain the low yield of aldehyde obtained.

The above considerations indicate the structure of a 4-acetamido-2-amino-2, 4, 6-trideoxy-hexose for Compound A and the derived structures for its N-acetyl derivative (Compound B) and for the diamino sugar obtained by strong acid treatment (Compound C) (Fig. 2). Although this latter compound has not been isolated in pure form, its behavior on paper chromatograms is compatible with the proposed structure. It gives the typical reactions of a 2-amino-2-deoxy sugar, and moves on paper with an Rf close to those of glucosamine and galactosamine (Table I). This rate of movement may be explained by the compensating effects of a methyl group, which increases chromatographic mobility, and of an additional amino group, which decreases it (14, 29).

The behavior of Compound A on paper chromatograms is also consistent with the data reported for other substituted sugars, in which replacement of one hydroxyl group by an amino group salified by hydrochloric acid decreases the rate, and replacement by an acetylamino group increases it (14, 29). The fast movement of Compound A would be the combined result of two rate increasing groups (acetylamino and methyl) and one rate decreasing group (amino hydrochloride). The very slow elution rate from the column of the sulfonic resin Dowex 50 can be explained by the reverse effect of the above mentioned groups. Crumpton has already reported the strong retarding effect of the methyl group in N-fucosamine on its movement on ion exchange columns under similar conditions (30).

Degradation of Compound A with ninhydrin affords a compound possessing structure D (Fig. 2). Its high mobility on paper chromatography is consistent with the type of structure proposed. Furthermore, the presence of a pair of free hydroxyls at C-2 and C-4 is favorable for complex formation with borate (31), in agreement with its fast movement on paper electrophoresis. In addition, compounds which have this structure do not give a positive reaction with the aniline phthalate reagent (32), and this observation was verified experimentally with the ninhydrin degradation product of Compound A.

The presence of a diaminohexose component in a bacterial polysaccharide seems never to have been reported before. Even if it is too early to ascertain its biological implication, it is of interest to note that the only other evidence for the existence of diamino sugars in nature has been found in the low molecular weight and framycetin (36). Recent work on the structure of these compounds, however, has estab-
lished a structure quite different from the one proposed in the present work (37, 38).

SUMMARY

A new amino sugar was found in acid hydrolysates of a polysaccharide from Bacillus subtilis. It was isolated by the use of ion exchange chromatography, and obtained in a pure crystalline form. Its composition corresponds to $\text{C}_9\text{H}_{18}\text{O}_6\text{N}_2\text{Cl}$. On the basis of color reactions, periodate oxidation studies, and infrared spectra, it is proposed that the new compound is a 4-acetamido-2-amino-2,4,6-trideoxy-hexose.

REFERENCES

The Diaminohexose Component of a Polysaccharide Isolated from Bacillus subtilis
Nathan Sharon and Roger W. Jeanloz


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