Biosynthesis of Streptococcal Cell Walls: 
N-Acetyl-D-Muramic Acid

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Glucose-1-14C and acetylglucosamine-1-14C were added singly and together with equal amounts of the unlabeled reciprocal to Brain Heart Infusion and used for the culture of Streptococcus pyogenes. The labeling pattern of the rhamnose, glucosamine, and muramic acid in the cell wall supported an intermediary role for acetylglucosamine in providing the C1-C6 moiety of muramic acid. Although radioactivity in the C2-C9 portion of muramic acid suggested that some of the lactyl group (C7-C9) came from glycolytic products, there was also considerable contribution to it from noncarbohydrate sources. Using cell-free extracts, we were unable to demonstrate biosynthesis of acetylmuramic acid, either free or nucleotide-bound, while glycolysis was occurring. The formation of uridine diphosphoacetylmuramic acid has been reported by others who used uridine diphospho-N-acetyl-D-glucosamine, phosphoenolpyruvate, reduced nicotinamide adenine dinucleotide, and reduced nicotinamide adenine dinucleotide phosphate. However, we did not detect the formation of this compound.

Decisive evidence for the pathway(s) of biosynthesis of acetylmuramic acid by microorganisms has been lacking. O'Brien, Glick, and Zilliken (6) have demonstrated that the N-acetyl-D-glucosamine moiety of 2,β-methyl-N-acetyl-D-glucosaminide is utilized by Lactobacillus bifidus var. pennsylvanicus without significant dilution to form muramic acid. Since the specific activity per mole was the same in the isolated muramic acid as in the glucosaminide, it seemed probable that only the ring was labeled, and that the side chain originated from unlabeled components of the growth medium which presumably contained lactose, amino acids, and a vitamin supplement. Subsequent experiments by Richmond and Perkins (8), using Staphylococcus aureus and labeled D-glucose, supported the concept of a precursor relationship of N-acetyl-D-glucosamine to muramic acid, although the experimental design used did not permit a definitive answer. It seemed clear, however, that the side chain of muramic acid originated largely from glycolytic products. These findings were interpreted as agreeing with the results of earlier experiments of Strominger (13). His experiments with cell-free extracts of S. aureus, Escherichia coli, and Aerobacter aerogenes indicated a condensation of uridine diphospho-N-acetyl-D-glucosamine (UDP-GNAc) with phosphoenolpyruvate (PEP) to yield a 3-O-enolpyruvyl-N-acetyl-D-glucosamine-nucleotide.

Earlier work in this (10) and other laboratories (2, 9) had demonstrated that glucose is converted by Streptococcus pyogenes to D-glucosamine and L-rhamnose without scission of its carbon skeleton. Glucose in the medium serves principally as an energy source through glycolysis, resulting in its conversion to lactic acid. Our subsequent finding (14), that these organisms utilize a single hexokinase to phosphorylate N-acetyl-D-glucosamine and D-glucose, led to the demonstration that either carbohydrate could be utilized, without adaptation, for supporting growth in a complex medium. Therefore, the group A streptococci appeared useful for investigating pathways of muramic acid biosynthesis. A preliminary report of our findings has been presented (S. S. Barkulis et al., Bacteriol. Proc., p. 99, 1966).

MATERIALS AND METHODS

S. pyogenes, serotype 14, was grown in Difco Heart Infusion Broth supplemented with salts and either D-glucose or N-acetyl-D-glucosamine at 1.0% or with both at 0.5% each. Cells were harvested in late log phase (10 hr), washed twice in distilled water, and disintegrated in a Shockman shaker. Cell walls were isolated as previously described (1). They were then treated with trypsin to remove the type-specific

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protein components. The walls were hydrolyzed in 2 N HCl for 2 hr in a sealed tube at 100 °C. The acid was removed in vacuo and the dried material was dissolved and applied to a column of Dowex 50W × 2 in hydrogen form. Neutrals were eluted with water and the bases were eluted with 2 N HCl. The basic fraction was further hydrolyzed for 10 hr in 4 N HCl. After removal of acid, the muramic acid and glucosamine of the hydrolysate were separated by the method of Park as described by Perkins and Rogers (7).

Rhamnose was isolated from the neutral fraction by paper chromatography in butanol-acetic acid-water (62:15:25) and was quantitated by the procedure of Dische and Shetlles (4); its radioactivity was then determined. Glucosamine and muramic acid were quantitated by the Elson-Morgan procedure as modified by Boas (3). Their radioactivity was determined after paper chromatography in butanol-pyridine-water-acetic acid (60:40:30:3) and paper electrophoresis in 2 N acetic acid at 900 v for 2.5 hr. The compounds were eluted from paper with water; the samples were dried and then reconstituted to a definite volume for analysis.

RESULTS

Table 1 summarizes the principal findings. Glucose was utilized with little dilution for the biosynthesis of cell wall rhamnose and glucosamine. Muramic acid contains a small but significant excess of radioactivity compared to glucose. When equal amounts of unlabeled acetylglucosamine were present in the medium (Table 1, line 2), there was only a small dilution of glucose carbon which went to rhamnose. However, the added acetylglucosamine almost completely preempted the pathway by which glucosamine is incorporated into the cell wall and markedly diluted glucose carbon furnished for muramic acid. The latter contained substantial radioactivity. These findings, which support an intermediary role for acetylglucosamine in muramic acid biosynthesis in these bacteria, suggested to us that C1–C6 of glucosamine becomes the corresponding carbon moiety of muramic acid, and that a product of glycolysis supplies, in part, the carbon for the side group (C7–C9) of muramic acid.

This conclusion was strengthened by the results obtained when acetylglucosamine-[1-14C] was used as a carbohydrate source. It is apparent from comparing lines 1 and 3 of Table 1 that the labeling pattern in the cell wall constituents is similar to either carbohydrate as the principal substrate. The results shown in line 4 are complementary to those in line 2. Added cold glucose again preferentially supplies the carbon skeleton of rhamnose with acetylglocosamine, providing the principal carbon for both cell wall glucosamine and muramic acid. The decrease in radioactivity of muramic acid from line 3 to line 4 is significant.

To obtain further evidence that the lactyl group of muramic acid (C7–C9) was formed to some degree from glycolytic products, the muramic acid shown in line 3 was degraded. Table 2 summarizes these results. When C1 was removed with ninhydrin, by using the procedure of Stoffyn and Jeanloz (11), the resulting carbon skeleton of muramic acid (C2-C9) contained 21% of the radioactivity of the substrate acetylglucosamine-[1-14C]. To obtain a specific activity of the C2–C9 carbon skeleton, the product, after a ninhydrin degradation, was further degraded with sulfuric acid by the method of Strange and Kent (12) and the liberated acetaldehyde was quantitated. The acetaldehyde, representing C8-C9 of muramic acid, contained a substantial part of the total radioactivity of C2-C9. However, there was insufficient material to permit an accurate determination of the specific activity of this product.

The degradation of muramic acid, therefore, indicated that the radioactivity of GNAc-[1-14C] was largely present in C1 of muramic acid. The remainder of the molecule (C2-C9) contained roughly the expected amount of radioactivity predicted from data in lines 1 and 3 of Table 1, with most of the radioactivity in C7-9. If a product in the glycolytic pathway from phosphoglyceric acid to lactic acid had supplied the side

### Table 1. Labeling of streptococcal wall constituents in cells grown on glucose-[1-14C] or GNAc-[1-14C]

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Relative specific activity (muc/mole)*</th>
<th>Rhamnose</th>
<th>Glucosamine</th>
<th>Muramic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose-[1-14C]</td>
<td>—</td>
<td>0.94</td>
<td>0.92</td>
<td>1.16</td>
</tr>
<tr>
<td>Glucose-[1-14C]</td>
<td>GNAc</td>
<td>0.82</td>
<td>0.02</td>
<td>0.25</td>
</tr>
<tr>
<td>GNAc-[1-14C]</td>
<td>—</td>
<td>0.80</td>
<td>1.02</td>
<td>1.15</td>
</tr>
<tr>
<td>GNAc-[1-14C]</td>
<td>Glucose</td>
<td>0.10</td>
<td>0.99</td>
<td>0.89</td>
</tr>
</tbody>
</table>

* Specific activity of glucose-[1-14C] was 1.07 and of GNAc-[1-14C] was 5.17. Each was set equal to unity for tabulation, and specific activity of the cell wall sugars was divided by specific activity of the respective substrates to permit easier comparison.

### Table 2. Determination of radioactivity in muramic acid

<table>
<thead>
<tr>
<th>Substance</th>
<th>Relative specific activity (muc/mole)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>GNAc-[1-14C]</td>
<td>1.00*</td>
</tr>
<tr>
<td>Muramic acid (C1-C9)</td>
<td>1.15</td>
</tr>
<tr>
<td>Muramic acid (C2-C9)</td>
<td>0.21</td>
</tr>
</tbody>
</table>

* Specific activity set equal to unity for tabulation; actually 5.17. See footnote of Table 1.
chain of muramic acid without dilution, C2-C9 should have had 50% of the activity of either glucose or acetylglucosamine. In our experiments, considerably less was consistently obtained, indicating either a biosynthetic pathway not involving glycolytic products or a large dilution of them.

**DISCUSSION**

Extracts of *S. pyogenes* are unable to phosphorylate either muramic acid or acetylglucosamine. They do contain uridine diphosphoacetyl-muramic acid (UDPMurAc) and are able to synthesize UDPMurAc-peptides (Barkulis et al., in preparation). Our data, therefore, were consistent with the possibility that UDPMurAc is synthesized at the nucleotide level from UDPGNAc and a three-carbon product of glycolysis; Fig. 1, representing a compilation of reported pathways, could account for the data presented.

We have been unable to demonstrate synthesis of UDPMurAc in streptococcal extracts prepared and fortified in a variety of ways calculated to examine the pathways shown in Fig. 1. In particular, actively glycolyzing extracts have been supplemented with UDPGNAc, and many other possible cosubstrates. The conditions utilized by Strominger have also been examined. To date, however, we have not been successful. It has been suggested (C. W. Shuster and J. Betts, Bacteriol. Proc., p. 77, 1966) that pyruvate is the immediate precursor of the three-carbon strain. Their studies were with *Pseudomonas saccharophila*. After our manuscript was completed, Gunetileke and Anwar (5) presented convincing evidence that extracts of *Aerobacter cloacae* synthesized UDPMurAc from UDPGNAc, PEP, and reduced nicotinamide adenine dinucleotide phosphate. Our failure to detect this reaction, therefore, may have been due to inadequate techniques or may indicate still another pathway for acetylmuramic acid synthesis. In view of the many variations that have now been found in the complex position and structure of the mucoprotein component of the walls of different species and genera of bacteria, it is not unlikely that alternative ways may exist for synthesizing one of its principal constituents.

**LITERATURE CITED**


