Biosynthesis of the Polysaccharide of Micrococcus lysodeikticus Cell Walls

I. CHARACTERIZATION OF AN IN VITRO SYSTEM FOR POLYSACCHARIDE BIOSYNTHESIS

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SUMMARY

The particulate enzyme fraction obtained from Micrococcus lysodeikticus catalyzed the incorporation of D-[14C]glucose from uridine diphospho-D-[14C]glucose into a polymer which resembled the D-glucose- and N-acetyl-D-mannosaminuronic acid-containing polysaccharide of the cell walls of this organism. In vitro biosynthesis of the polysaccharide depended upon the presence of the uridine diphospho-N-acetylmuramiduronic acid fraction isolated from M. lysodeikticus and uridine diphospho-N-acetyl-D-glucosamine and was stimulated by a heat-stable factor obtained from the soluble fraction of cell extracts. The reaction proceeded optimally at pH 8.2 and at 20 mM magnesium ion concentration. The incorporation of D-[14C]glucose proceeded after a lag period of 15 to 20 min. Preincubation of the reaction components with the particulate enzyme fraction before the addition of uridine diphospho-D-glucose eliminated the lag period.

[14C]N-Acetylmuramiduronic acid residues were incorporated into polysaccharide from [14C]uridine diphospho-N-acetylmuramiduronic acid (labeled throughout the molecule) in an amount approximately equimolar to D-glucose in a reaction dependent upon uridine diphospho-D-glucose. However, when uridine diphospho-N-[14C]acetyl-D-glucosamine was the labeled substrate, N-[14C]acetyl-D-glucosamine was incorporated only to the extent of 1 residue or less for each 15 residues of D-glucose.

Cell walls of Micrococcus lysodeikticus contain two major polymers. Peptidoglycan, the more abundant polymer, has been extensively studied with respect to its structure (1, 2) and its biosynthesis (3-5). Perkins (6) extracted the other polymer from M. lysodeikticus cell walls with trichloroacetic acid and established that it was a polysaccharide containing D-glucose and N-acetylmannosaminuronic acid (2-acetamido-2-deoxymannuronic acid) in approximately equal amounts. The polysaccharide may consist of alternating residues of glucose and N-acetylmannosaminuronic acid. The results of fractionation and analyses of M. lysodeikticus cell walls by Jeanloz (7) suggested that glucose is more abundant than the N-acetylmannosaminuronic acid.

The glucose residues of the polysaccharide are substituted at position 6 and are destroyed by periodate, whereas the N-acetylmannosaminuronic acid residues are not affected by periodate (8). The N-acetylmannosaminuronic acid residues occupy internal positions of the polysaccharide chain, are of the p-configuration, and are substituted at position 4 (9, 10).

When the polysaccharide was isolated from cell walls solubilized by the action of lysozyme or by lysozyme in combination with Streptomyces ML endopeptidase and N-acetylmuramyl-L-alanine amidase, it was shown to contain glucose and N-acetylmannosaminuronic acid and, in addition, smaller quantities of muramic acid phosphate, peptidoglycan peptide subunits, and glucosamine (2). In view of the specificities of the enzymes used, the low molar ratios of phosphate and peptidoglycan components reported relative to glucose (approximately 0.1) suggest that these preparations included not only the polysaccharide but also the linkage region by which the polysaccharide is covalently attached to peptidoglycan in native cell walls. In this respect the D-glucose- and N-acetyl-d-mannosaminuronic acid-containing polysaccharide of M. lysodeikticus appears to resemble the teichoic acids of Staphylococcus aureus (11), the teichoic acid and the teichuronic acid of Bacillus licheniformis (12, 13), the C and G polysaccharides of Streptococcus pyogenes (14), and the C-specific polysaccharide of Lactobacillus casei (15), all of which appear to be covalently linked to peptidoglycan through a phosphodiester bond to a N-acetylmuramic acid residue.

Rosenthal and Sharon (16) isolated a nucleotide sugar from M. lysodeikticus which was later identified as uridine diphospho-N-acetyl-d-mannosaminuronic acid (17). Biely and Jeanloz (18) also purified a nucleotide sugar from the same organism and established it to be UDP-N-acetyl-d-glucosaminuronic acid. According to a brief report (19), UDP-GlcNAcUA and UDP-

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1 The abbreviations used are: UDP-GlcNAcUA, uridine diphosphono-N-acetyl-d-glucosaminuronic acid; UDP-Glc, UDP-N-glucose; UDP-Nea-NAcUA, UDP-N-acetylmannosaminuronic acid; UDP-
UDP-d-glucose are substrates for in vitro synthesis of the *M. lysodeikticus* polysaccharide.

In this paper we report the characteristics of a system in which a particulate enzyme preparation from *M. lysodeikticus* requires UDP-Glc, the UDP-N-acetylhexosaminuronic acid fraction isolated from the same microorganism, and UDP-GlcNAc, as well as a heat-stable factor for the in vitro biosynthesis of a polymer similar to the *N*-glucose- and *N*-acetyl-D-mannosaminuronic acid-containing polysaccharide present in the cell walls of this microorganism. The following paper (20) reports that the UDP-HexNAcUA fraction contains UDP-ManNAcUA, which is the donor of the *N*-acetyl-D-mannosaminuronic acid residues of the polysaccharide.

**EXPERIMENTAL PROCEDURE**

**Materials and Microorganisms--**Most of the UDP-[U-14C]Glc used in the experiments reported below was prepared enzymatically from d-[U-14C]glucose in about 60% yield essentially as described by Thomas et al. (21). Some UDP-[U-14C]Glc (194 mCi per mmole) was obtained from Nuclear-Chicago and the remainder from Calbiochem (35 mCi per mmole). UDP-GlcNAc labeled in the acetyl group was prepared enzymatically from [1-14C]acetate with partially purified extracts from *Saccharomyces cerevisiae* by procedures similar to those employed by Nathenson and Strominger (22). Both UDP-[14C]Glc and UDP-GlcNAc were purified by paper chromatography in Solvent A (isobutyric acid-1 M ammonium hydroxide (5:3, v/v)) and Solvent B (95% ethanol-1 M ammonium acetate, pH 7 (15:6, v/v)), after which the compounds recovered were free of radioactive contaminants and did not contain detectable amounts of other ultraviolet-absorbing compounds. The UDP-[14C]Glc was characterized by its ultraviolet absorption spectrum, by chromatographic comparison with authentic UDP-Glc, and by the use of venom phosphodiesterase and alkaline phosphatase to release d-[14C]glucose which was identified by quantitative conversion to d-[14C]glucoselactone by glucose oxidase. Similarly, UDP-GlcNAc was characterized by its ultraviolet absorption spectrum, by chromatographic comparison with authentic UDP-GlcNAc, and by the use of venom phosphodiesterase to release d-[14C]glucose which was identified by quantitative conversion to d-[14C]glucoselactone by glucose oxidase.

Cultures of *Micrococccus lysodeikticus* ATCC 4698 were kindly provided by Dr. Martin Dworkin, Department of Microbiology, University of Minnesota, Minneapolis, and by Dr. Wilmar L. Sano, Department of Chemistry, University of Minnesota, Duluth. The cultures were maintained on agar slants of 1% Bacto-peptone and 0.5% NaCl with monthly transfers.

**Preparation of Particulate Enzyme--**Liquid cultures of *M. lysodeikticus* were grown with gyratory shaking at 37° in 1% Bacto-peptone and 0.5% NaCl (24) with a doubling time of about 21 hours. Cells were harvested by centrifugation from cultures at mid-log phase as determined by turbidimetric measurement at 700 nm. Centrifugation and all subsequent operations were at 0-4°. The cells from 6 liters of culture were washed in 100 ml of 0.5% NaCl, centrifuged by centrifugation, washed in 100 ml of 0.05 M Tris-HCl, pH 8.2, 0.1 M MgCl₂, 2 mM 2-mercaptoethanol (TMM buffer), and collected again by centrifugation. The pellet of cells, approximately 9 g wet weight, was transferred to a previously cooled mortar and ground with 27 g of alumina. Vigorous grinding was continued for 5 to 10 min after the cells and alumina had reached a uniform consistency of a very heavy paste. The paste was suspended in 100 ml of TMM buffer and centrifuged for 5 min at 2,000 × g to remove most of the alumina. The supernatant fluid was centrifuged at 12,000 × g for 10 min to sediment intact cells, cell walls, and residual alumina. This centrifugation was repeated to remove residual cell walls and debris. The supernatant fluid was then centrifuged for 1 hour at 105,000 × g in the No. 40 rotor of the Spincoc model L ultracentrifuge. The supernatant solution (S-100) was decanted and retained for subsequent use. The residue was washed by suspension in about one-half of the original volume of TMM buffer and recentrifuged at 105,000 × g for 1 hour. The pellet was suspended in 3.0 ml of TMM buffer to give a final protein concentration of about 10 mg per ml as determined by the procedure of Lowry et al. (23). This particulate enzyme preparation was stored at -15° with only slight loss of activity over a period of several weeks.

**Preparation of Heated S-100--**Two alternative procedures were followed in the preparation of the heated supernatant fraction; both employed the S-100 obtained during the preparation of the particulate enzyme fraction as described above. In Procedure A, a small volume of the S-100 was heated for 10 min in a boiling water bath. There was no evidence of the formation of any precipitate. In the alternate method, Procedure B, which was used more frequently because of the apparent purification which it effected, 4 volumes of S-100 were mixed with 1 volume of a solution of 0.10 M magnesium acetate, either 0.25 M Tris-HCl or HEPES of pH 8.2, and 0.025 M 2-mercaptoethanol and were heated for 10 min in a boiling water bath. The large amount of precipitate which formed was removed by centrifugation at 12,000 × g for 10 min. The resulting faintly yellow supernatant solution was routinely used in the assay mixtures for polysaccharide biosynthesis as described below. No means has been established for measuring the quantity of the factor present other than its activity of promoting polysaccharide biosynthesis since the chemical nature of this factor is still unknown. Most assay mixtures (1.0 volume) received 0.1 volume of the heated S-100 fraction.

**Isolation of UDP-HeXNAcUA--**UDP-HeXNAcUA cells harvested from 12 liters of late log phase cultures were promptly resuspended in 12 liters of fresh medium containing 0.2 g per liter each of glycine, DL-alanine, DL-glutamic acid, and L-lysine hydrochloride, 10 g per liter of d-glucose, 50 mg per liter of chloramphenicol, and 10 mg per liter of penicillin G. In 0.05 M potassium phosphate buffer of pH 7.0 with 0.001 M EDTA (26). Incubation was continued for 75 min at 37° with gyratory shaking. The cells were harvested and washed in 0.5% NaCl. The cells (22 g wet weight) were suspended and stirred in 100 ml of 10% triethanolamine acid for 90 min at 0°, at which time the extracted
cells were removed by centrifugation. The trichloroacetic acid was removed from the supernatant fluid by five solvent extractions each with 1 volume of diethyl ether. In an alternative procedure the cells were extracted by heating a water suspension of cells at 100°C for 10 min and then were centrifuged to remove cell debris. The extract prepared by either method was adjusted to pH 5 with NH₄OH, concentrated to about 3 ml by rotary evaporation (35°C bath), and applied to four sheets of Whatman No. 3MM filter paper with reference samples of UDP-GlcNAc and UDP-HexNAcUA. The paper chromatograms were developed by descending chromatography for 36 hours in isobutyric acid-1 m NH₄OH (5:3, v/v) and washed three times with water. The ultraviolet-absorbing band of material with a mobility corresponding to the reference UDP-HexNAcUA \((\text{R}_{\text{UDP-HexNAcUA}})\) of 0.8) was cut from the chromatogram and recovered from the paper by descending elution with water. The recovered material was applied to two sheets of Whatman No. 3MM filter paper with reference compounds and developed in 95% ethanol-1 m ammonium acetate, pH 7 (15:6, v/v) for 24 hours, washed twice with 95% ethanol and once with acetone. The ultraviolet-absorbing band of material with a mobility corresponding to UDP-HexNAcUA \((\text{R}_{\text{UDP-HexNAcUA}})\) of 0.7) was eluted with water. Since additional purification was usually necessary, the sample was subjected to electrophoresis on Whatman No. 3MM paper in 0.15 m triethylammonium acetate, pH 4.4, at 3000 volts for 45 min. The ultraviolet-absorbing band of material corresponding to UDP-HexNAcUA \((\text{R}_{\text{UDP-HexNAcUA}})\) of 1.3) was eluted and rechromatographed in the isobutyric acid-ammonium hydroxide system as described above. The purified material (about 20 μmoles) had the ultraviolet absorption spectrum of a uridine nucleotide. It did not contain detectable amounts of contaminants as determined by absorption of ultraviolet light following paper chromatography and electrophoresis. Characterization of the preparation of UDP-HexNAcUA is reported in the accompanying paper (20).

**Isolation of [14C]UDP-HexNAcUA—Cells of M. lysodeikticus harvested at mid-log phase were resuspended in 1 volume of artificial medium containing L-alanine, L-glutamic acid, L-lysine hydrochloride, and glycine (each 0.2 mg per ml), glucose (50 μg per ml), and potassium phosphate (0.05 m, pH 7.0). Fifty microliters of [14C]glucose were added to 10 ml of the cell suspension in artificial medium. Chloramphenicol (final concentration, 50 μg per ml), penicillin G (10 μg per ml), and EDTA (1 mM) were added after 10 min of shaking at 37°C. After 10 additional min of incubation, unlabeled glucose was added to bring the glucose concentration of the medium to 500 μg per ml. After a total of 110-min incubation with the radioactive glucose, the cells were harvested and the nucleotides were extracted with trichloroacetic acid and purified by paper chromatography in isobutyric acid-ammonium hydroxide and in the ethanol-ammonium acetate solvents as described above for the preparation of unlabeled UDP-HexNAcUA. The purified radioactive material had the ultraviolet absorption spectrum of an uridine nucleotide and was indistinguishable from UDP-HexNAcUA by paper chromatography. The purified sample contained 75 nmoles of [14C]UDP-HexNAcUA with a specific activity of 4700 cpm per nmole.

The distribution of radioactivity in [14C]UDP-HexNAcUA (labeled throughout the molecule) was determined by treatment with venom phosphodiesterase and separation of the reaction products by paper chromatography in the ethanol ammonium acetate solvent. The N-acetylhexosaminuronic acid 1-phosphate contained 60.5% of the total radioactivity and the remainder was found in UMP (39.4%). When [14C]UDP-HexNAcUA was treated with both venom phosphodiesterase and alkaline phosphatase, 56.4% of the radioactivity was found in the N-acetylhexosaminuronic acid and 43.6% in uridine. For calculation purposes, the monosaccharide moiety was assumed to contain 58.5% of the total radioactivity in [14C]UDP-HexNAcUA.

**Assay Procedure for Polysaccharide Synthesis—Routine assay mixtures contained either 0.2 or 0.4 mM UDP-[14C]Glc (3 or 7 x 10⁶ cpm per nmole), 0.4 mM UDP-HexNAcUA, 0.4 mM UDP-GlcNAc, 50 mM HEPES buffer adjusted to pH 8.2 with NaOH, 20 mM MgCl₂, 5 mM 2-mercaptoethanol, 0.1 volume of heated S-100 of M. lysodeikticus, and particulate enzyme at a final protein concentration of about 1 mg per ml. In conventional experiments, reaction mixtures were incubated at 37°C for the prescribed period of time. In experiments involving preincubation, reaction mixtures complete except for UDP-[14C]Glc were preincubated for 30 min at 37°C; at which time the UDP-[14C]Glc was added and the incubation continued. Aliquots (20 μl) of reaction mixtures removed during the incubation period were inactivated by addition to an equal volume of solution of isobutyric acid-1 m NH₄OH (5:3, v/v).

Inactivated reaction aliquots were applied quantitatively to Whatman No. 3MM filter paper and subjected to descending chromatography in isobutyric acid-1 m NH₄OH (5:3, v/v) for 6 to 10 hours. The distribution of radioactive compounds along the channel of solvent development was determined with a Packard model 7201 radiochromatogram scanner. On the basis of the scan the radioactive portions corresponding to the origin, UDP-Glc, and glucose phosphate were cut out and transferred to scintillation vials for quantitative evaluation in a Packard model 3525 liquid scintillation spectrometer. The scintillation mixture contained 4 g of 2,5-diphenyloxazole and 0.5 g of 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene in 1 liter of toluene.

The amount of radioactivity retained at the origin of the chromatogram indicated the extent of incorporation of the radioactive substrate into polysaccharide. It should be noted that summation of the radioactivity detected on all the paper chromatogram segments (origin, UDP-Glc, and glucose phosphate were cut out and transferred to scintillation vials for quantitative evaluation in a Packard model 3525 liquid scintillation spectrometer. The scintillation mixture contained 4 g of 2,5-diphenyloxazole and 0.5 g of 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene in 1 liter of toluene. The amount of radioactivity retained at the origin of the chromatogram indicated the extent of incorporation of the radioactive substrate into polysaccharide. It should be noted that summation of the radioactivity detected on all the paper chromatogram segments (origin, UDP-Glc, and glucose phosphate) gave a highly reproducible recovery of radioactivity. The percentage of mean deviation of summed radioactivity in aliquots taken from a common reaction mixture was almost always less than ±1.0%.

**RESULTS**

**General Requirements for Polysaccharide Synthesis—**The initial demonstration of in vitro biosynthesis of the M. lysodeikticus cell wall polysaccharide consisting of n-glucose and N-acetyl-n-mannosaminuronic acid was dependent upon the presence of the appropriate precursor nucleotides, the preparation of a suitable enzyme fraction, and the selection of appropriate reaction conditions. UDP-Glc was selected as the most probable donor of n-glucose residues. The availability of n-[14C]glucose-labeled UDP-Glc permitted the development of an assay procedure, based on the incorporation of radioactive glucose into a chromatographically immobile product, analogous to an assay procedure developed previously for the study of in vitro peptidoglycan biosynthesis (8). The donor of N-acetyl-n-mannos-
ticulate fraction, and is stimulated by a heat-stable factor which and UDP-GlcNAc, is catalyzed by the enzymes of the par- 


Omission of the particulate enzyme completely eliminated prod-

tuct formation as did the substitution of heat-treated particulate enzyme in place of the active preparation of particulate enzyme. A control reaction mixture, in which all nucleotide sugars were added after the inactivation of the venom phosphodiesterase, could not replace the latter as a donor of glucose residues. Similarly, \( \alpha \)-N-acetylgalactosaminuronic acid 1-phosphate and or-N-acetyl-\( \alpha \)-glucosamine 1-phosphate could not substitute for UDP-HexNAcUA and UDP-GlcNAc, respectively, in their ability to support product formation. A

with snake venom phosphodiesterase to release UMP and the corresponding monosaccharide-1-phosphate. Following inac-
tivation of the venom phosphodiesterase, the remaining com-
ponents of the assay reaction mixtures were added, and the incubations were continued for 2 hours at 37°. Preliminary treatment of each of the nucleotide sugars with venom phosphodiesterase abolished polysaccharide synthesis (Table II). \( \alpha \)-B-\([\text{C}]\)Glucose 1-phosphate, formed from UDP-\([\text{C}]\)Glc by the venom phosphodiesterase, could not replace the latter as a donor of glucose residues. Similarly, \( \alpha \)-acetylhexosaminuronic acid 1-phosphate and \( \alpha \)-N-acethyl-\( \beta \)-glucosamine 1-phosphate could not substitute for UDP-HexNAcUA and UDP-GlcNAc, respectively, in their ability to support product formation. A

Control reaction mixture, in which all nucleotide sugars were added after the inactivation of the venom phosphodiesterase, formed product to an extent comparable with that observed in the reaction mixture not treated with venom phosphodiesterase.

**Table I**

<table>
<thead>
<tr>
<th>Additions and deletions</th>
<th>Glucose incorporated (nmoles)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete</td>
<td>1.24</td>
</tr>
<tr>
<td>Minus UDP-HexNAcUA</td>
<td>0.00</td>
</tr>
<tr>
<td>Minus UDP-GlcNAc</td>
<td>0.12</td>
</tr>
<tr>
<td>Plus UDP-MurNAc-pentapeptide</td>
<td>0.83</td>
</tr>
<tr>
<td>Minus particulate enzyme fraction</td>
<td>0.65</td>
</tr>
<tr>
<td>Minus particulate enzyme fraction plus heated particulate enzyme fraction</td>
<td>0.05</td>
</tr>
<tr>
<td>Minus S-100</td>
<td>0.63</td>
</tr>
<tr>
<td>Minus S-100 plus heated S-100 (Procedure A)</td>
<td>1.00</td>
</tr>
</tbody>
</table>

**Table II**

<table>
<thead>
<tr>
<th>Substrate previously treated with venom phosphodiesterase</th>
<th>Substrates added after inactivation</th>
<th>Glucose incorporated (nmoles)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>UDP-([\text{C}])Glc</td>
<td>1.05</td>
</tr>
<tr>
<td></td>
<td>UDP-HexNAcUA</td>
<td>UDP-GlcNAc</td>
</tr>
<tr>
<td>UDP-([\text{C}])Glc</td>
<td>UDP-HexNAcUA</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>UDP-GlcNAc</td>
<td></td>
</tr>
<tr>
<td>UDP-HexNAcUA</td>
<td>UDP-([\text{C}])Glc</td>
<td>0.11</td>
</tr>
<tr>
<td></td>
<td>UDP-HexNAcUA</td>
<td>UDP-GlcNAc</td>
</tr>
<tr>
<td>Buffer</td>
<td>UDP-([\text{C}])Glc</td>
<td>1.25</td>
</tr>
<tr>
<td></td>
<td>UDP-HexNAcUA</td>
<td>UDP-GlcNAc</td>
</tr>
</tbody>
</table>

* A conventional reaction mixture; no venom phosphodiesterase was added at any stage of the assay.

* Only the reaction buffer was previously treated with venom phosphodiesterase.
lag period was eliminated by preincubation, reaction mixtures for a reaction mixture which was preincubated for 30 min in the case. Fig. 2 shows the time course of $[^{14}C]glucose$ incorporation. Thus a reaction mixture containing all substrates except UDP-$[^{14}C]Glc$ might incorporate $[^{14}C]glucose$ without delay. Such was found to be the case. The requirement for magnesium ions was determined in a similar fashion. 

A rather striking lag period of about 15 to 20 min was noted for the complete reaction mixture shown in Fig. 1. The other reaction mixture, which contained the same reaction components with the exception of UDP-$[^{14}C]Glc$, was preincubated for 30 min at 37°C at which time (abscessa = 0 min) UDP-$[^{14}C]Glc$ was added and the incubation continued. Fig. 2 shows the time course of $[^{14}C]glucose$ incorporation for a reaction mixture which was preincubated for 30 min prior to the addition of UDP-$[^{14}C]Glc$. Product formation began immediately after the addition of UDP-$[^{14}C]Glc$ in contrast to the control reaction which was not preincubated. Since the lag period was eliminated by preincubation, reaction mixtures were preincubated for 30 min prior to the addition of UDP-$[^{14}C]Glc$ in subsequent assays of product formation.

### pH, Metal Ion, and Substrate Concentration Requirements—
HEPES buffer was selected for pH control of the reaction system after difficulties were encountered with the Tris-HCl buffer which was used initially. When reaction mixtures prepared with Tris-HCl buffer were assayed by paper chromatography and the distribution of radioactivity was determined, as much as 50% of the total radioactivity was found to have a mobility about twice that of UDP-$[^{14}C]Glc$. It was ascertained that this material was a degradation product of UDP-$[^{14}C]Glc$ which was formed nonenzymatically during the application of the reaction mixtures to the paper chromatogram. The degradation product was presumed to be glucose 1,2-cyclic phosphate, since Paladini and Leloir (27) found that treatment of UDP-$[^{14}C]Glc$ with alkali yielded UMP and a cyclic phosphate ester of glucose, which they indicated was probably glucose 1,2-cyclic phosphate. Several factors were found to influence the extent of degradation, namely, Tris-HCl buffer of alkaline pH, the concentration of Mg$^{2+}$, and the amount of heat applied to hasten drying.

To determine the pH optimum, polysaccharide synthesis was measured in reaction mixtures containing HEPES buffer which were preincubated for 30 min. Fig. 3 is a plot of the extent of $[^{14}C]glucose$ incorporation 60 min after addition of UDP-$[^{14}C]Glc$ as a function of pH. The pH optimum is about 8.2.

The requirement for magnesium ions was determined in a similar fashion. Fig. 4 shows that the incorporation system has a rather sharp dependence on the concentration of magnesium ions. Maximum activity was obtained at a magnesium ion concentration of 20 mM although concentrations of 15 and 25 mM were nearly as effective. The divalent cations of manganese, calcium, cobalt, copper, iron, and zinc were tested at con-
A concentration of 0.4 mM was used to minimize variation in reaction velocity in subsequent assays for polysaccharide synthesis, all three substrates were added at concentrations of 0.4 mM. However, the concentration dependence for UDP-Glc is clearly different. Maximum reaction velocities are obtained when these substrates are present at concentrations of 0.4 mM. However, the concentration dependence for UDP-Glc is clearly different. Maximum reaction velocity is obtained with 0.2 mM UDP-Glc. In order to minimize variation in reaction velocity in subsequent assays for polysaccharide synthesis, all three substrates were added at a concentration of 0.4 mM.

**Particulate Enzyme and Heated Supernatant Factor Requirements**—The rate of product formation was directly proportional to the amount of particulate enzyme added to the reaction mixture (Fig. 5). The direct proportionality was observed not only with initial reaction rates, but also with the amount of product formed after extended periods of reaction.

As is shown in Table I, the polysaccharide synthesis system is stimulated by a component of the S-100. The requirement for S-100 could not be demonstrated with unwashed particulate enzyme preparations since a sufficient amount of the supernatant fraction remained as a contaminant of the particulate preparation. Different preparations of washed particulate enzyme have varied in their dependence upon heated S-100. The active component of heated S-100 recovered after passage through Sephadex G-25 was adsorbed to DEAE-cellulose at pH 8.2 and subsequently eluted with NaCl gradient. Unfortunately, the recovery of activity was rather low. The adsorption of the active component of heated S-100 was distributed primarily in the fractions between the major ultraviolet-absorbing materials. These results suggest that the active component is heterodisperse and of relatively large molecular weight.

The active component of heated S-100 recovered after passage through Sephadex G-25 was adsorbed to DEAE-cellulose at pH 8.2 and subsequently eluted with NaCl gradient. Fractions containing activity which stimulated polysaccharide biosynthesis were obtained at about 0.5 M NaCl. Unfortunately, the recovery of activity was rather low. The adsorption of the active component to DEAE-cellulose and the subsequent release of at least some of the component by salt suggest that it contains anionic functional groups.

In view of the requirement of the biosynthetic system for UDP-HexNAcUA and UDP-GlcNAc, it was of interest to determine whether the monosaccharide residues of either or both of these nucleotide sugars were incorporated into the reaction product.

Three polysaccharide synthesis assay mixtures were prepared in which the 14C-labeled substrate was either UDP-Glc, UDP-HexNAcUA, or UDP-GlcNAc. All were preincubated for 30 min in the absence of UDP-Glc. When UDP-Glc was added (unlabeled in two of the reaction mixtures and 14C-labeled in the other), incubation was continued and aliquots were removed periodically. Fig. 8 shows the results of these experiments. 14C-Glucose was incorporated rapidly during the incubation period. 14C-N-acetylmuramic acid was also incorporated rapidly in an approximately equal molar ratio with respect to glucose. The extent of 14C-N-acetylmuramic acid incorporation at the zero time point indicates that no more than 20% of N-acetylmuramic acid was incorporated during the 30-min preincubation period in the absence of UDP-Glc. In contrast, very little N-[14C]acetylglucosamine was incorporated into the product. In fact, the extent of N-acetylglucosamine incorporation was not distinguishable from the background for the assay procedure. This result has been confirmed several times. In no case has the incorporation of N-acetylglucosamine been such as to indicate clearly that it is a bona fide constituent of the reaction product. However, if the assumption is made that all of the radioactivity shown for the fractions assayed have been corrected for the amount of 14C-glucose incorporated in the absence of S.100 (0.067 molecule).

Properties of Reaction Product—Routine determination of synthesis of the polysaccharide was based on chromatographic immobility of the reaction product. Other experiments described below verified that the 14C-glucose-labeled reaction product is at least similar to, if not identical with, the polysaccharide found in cell walls of M. lysodeikticus.

14C-Glucose-labeled reaction product was prepared in a 1.0-ml reaction mixture which was preincubated without UDP-Glc, and then incubated with UDP-[14C]Glc. About 50% of the radioactive substrate was converted to product after 2 hours. The reaction mixture was diluted to 5 ml with water and centrifuged at 100,000 X g for 1 hour to sediment the particulate enzyme. Essentially all (90%) of the radioactive product was associated with the particulate enzyme. Lysozyme released only about 10% of the product from the particulate enzyme. However, the radioactive product was released from the particulate enzyme by a 10-min treatment in 0.05 M HCl at 75°. The released product was excluded from Sephadex G-75, indicating a molecular weight probably in excess of 50,000. However, this experiment does not distinguish a polysaccharide formed de novo from a polysaccharide in which a few residues of [14C]glucose were added to a previously existing acceptor already of high molecular weight.

Hydrolysis of radioactive product in 1 N HCl at 100° for 2 hours released a radioactive monosaccharide which had a chromatographic mobility identical with that of reference glucose and which was converted by glucose oxidase to a radioactive substance corresponding to d-gluconolactone. Hydrolysis of the [14C]glucose-labeled product in 1 N HCl at 100° for 30 min released about 10% of the initial radioactive material as an oligosaccharide. Although the characterization of the biosynthetic reaction product is incomplete, it appears to have the chemical properties expected of the n-glucose- and N-acetyl-D-mannosaminuronic acid-containing polysaccharide of M. lysodeikticus.

No Evidence for Lipid Intermediates—Since biosynthesis of peptidoglycan of M. lysodeikticus proceeds via the formation of lipid intermediates (4) in which the carrier lipid has been identified as the phosphomonoester of a C55 polyisoprenoid alcohol (29), and since the biosynthesis of a mannan of the cell membrane involves a similar carrier lipid intermediate (30-32), it was of interest to determine whether the biosynthesis of the d-glucose- and N-acetyl-D-mannosaminuronic acid-containing polysaccharide of the same organism might utilize a carrier lipid in a similar reaction pathway. Thus far we have not been able to obtain any evidence to support the participation...
of a carrier lipid in this biosynthetic reaction. When reaction mixtures prepared with UDP-[14C]Glc of very high specific activity (160 mCi per mmole) are chromatographed on paper in the isobutyric acid solvent, there is no detectable radioactivity in the region of the chromatogram expected to bear the supposed carrier lipid intermediates (RF = 0.8 to 0.9). Whereas the particulate enzyme fraction readily catalyzes the exchange of radioactivity of [14C]UMP with UDP-MurNAc-pentapeptide due to the reversibility of the reaction forming the first carrier lipid intermediate in peptidoglycan synthesis (4, 33), comparable assays failed to demonstrate any exchange of radioactivity of [14C]UMP with UDP-Glc, UDP-HexNAcUA, or with UDP-GlcNAc, either individually or in any possible combination.

**Discussion**

The results presented above clearly show that the particulate enzyme fraction obtained from *M. lysodeikticus* requires the three nucleotide sugars, UDP-Glc, the UDP-HexNAcUA fraction isolated from *M. lysodeikticus*, and UDP-GlcNAc for the formation of a chromatographically immobile reaction product. Glucose residues from UDP-Glc and N-acetylhexosaminuronic acid residues from UDP-HexNAcUA were incorporated into the product, but few or possibly no N-acetyl-d-glucosamine residues were incorporated from UDP-GlcNAc. The following paper (20) shows that the UDP-HexNAcUA fraction isolated from *M. lysodeikticus* contains two compounds, probably UDP-ManNAcUA and UDP-GlcNAcUA, and that the compound required for polysaccharide synthesis is UDP-ManNAcUA. Since cell walls of *M. lysodeikticus* have been shown to contain a polysaccharide consisting of approximately equal molar proportions of n-glucose and N-acetyl-d-mannosaminuronic acid (6, 9), it seems likely that the product formed in *vivo* is similar to, if not identical with, the cell wall polysaccharide. The presence of n-glucose in the reaction product has been established by identification with glucose oxidase following acid hydrolysis. [14C]UDP-HexNAcUA (labeled throughout the molecule) has not been available in sufficient quantity to prepare enough of the correspondingly labeled reaction product to attempt its characterization. A great deal of additional information about both the biosynthetic product and the native polysaccharide must be obtained to establish their structural identity.

The requirement of the polysaccharide-synthesizing system for a component of the S-100 varied with different preparations of particulate enzyme fraction. Some of the variations could be traced to incomplete removal of the soluble enzyme fraction from the particulate enzyme fraction. Unwashed particulate enzyme preparations showed no dependence on the supernatant factor. In contrast, the difference in degree of stimulation observed with washed particulate enzyme preparations was probably a function of the extent of contamination by a more tightly bound form of the active component.

Since the S-100 can be heated with relatively little loss of stimulatory activity, it seems unlikely that the active component is an enzyme. Rather extensive purification of the active component has been obtained through the heat precipitation of most of the protein of the S-100 followed by gel filtration. The active component is of rather high molecular weight since it is totally excluded from Sephadex G-25 and partially excluded from Sephadex G-200. Apparently it is anionic since it was adsorbed to DEAE-cellulose and eluted with 0.5 M salt. At the present time the active component of the S-100 has not been brought to a sufficient state of purity to obtain much meaningful information about its composition. The nonlinear response of the system for polysaccharide synthesis to supplementation by heated S-100 (Fig. 6B), together with the variation of particulate enzyme preparations in their degree of dependence on heated S-100, has hindered accurate quantitative evaluation of procedures employed to purify the active component. As is discussed below, the role of the active component of S-100 in the synthesis of polysaccharide may be that of an acceptor.

The requirement of the biosynthetic system for UDP-Glc and UDP-HexNAcUA, together with the observed incorporation of p-glucose and of N-acetylhexosaminuronic acid residues into reaction product in approximately equal molar amounts, corresponds well with the reported composition of the *M. lysodeikticus* cell wall polysaccharide. However, the obligatory requirement of the biosynthetic system for UDP-GlcNAc is not easily reconciled with the observation that little if any N-acetyl-p-glucosamine was incorporated into the product. In view of this apparent anomaly, several aspects of the experiments in which the extent of N-[14C]acetyl-p-glucosamine incorporation was determined should be considered. Since the UDP-GlcN[14C]Ac was labeled only in the acetyl group, it is conceivable that glucosamine was incorporated into the product with concomitant loss of the acetyl group. Although this possibility cannot be totally excluded at this time, it is not likely to be true, since no release of [14C]acetate was observed and since there was no change in the amount of residual radioactive UDP-GlcNAc even after extensive incubation with reaction conditions which formed large amounts of product when measured by [14C]glucose incorporation. The possibility of incorporation of glucosamine with loss of acetyl group could be clearly resolved with the use of UDP-GlcNAc labeled in the glucosamine moiety.

The standard assay procedure for polysaccharide synthesis is based on the amount of radioactivity which remains at the origin of paper chromatograms used to separate residual radioactive substrates from the reaction product. In the case of the experiments in which the radioactive substrate was UDP-GlcN[14C]Ac, the low level of radioactivity detected at the chromatogram origin was essentially independent of the length of incubation and was equivalent to the amount of radioactivity detected in the absence of one or both of the other substrates required for glucose incorporation. This amount of radioactivity may be merely the blank for the assay procedure, due to nonspecific adsorption of a small amount of radioactive substrate to the paper in the channel of solvent development or entrapment of substrate at the origin by the denatured particulate enzyme fraction. Alternatively, it might be due to enzymatic transfer of N-acetylglucosamine to endogenous acceptors quite apart from the synthesis of the p-glucose- and N-acetyl-d-mannosaminuronic acid-containing polysaccharide. Whatever the source or size of the assay blank, it should be noted that the only background value which has been subtracted from the data reported in this paper (except for Fig. 7) has been the background count rate of the liquid scintillation spectrometer and scintillation mixture.

An alternative explanation of the obligatory requirement for UDP-GlcNAc and the apparent low level of N-[14C]acetylglucosamine incorporation is related to the lag phenomenon observed in the time course of [14C]glucose incorporation in conventional reaction mixtures. As Fig. 2 clearly shows, preincubation of the reaction mixture lacking only UDP-Glc for 30 min prior to the addition of UDP-[14C]Glc eliminated the lag period. This result
indicates that at least two steps are involved in the pathway of polysaccharide synthesis \textit{in vitro} and that an early step is rate-limiting. Furthermore, the rate-limiting reaction proceeded in the absence of UDP-Glc, \textit{i.e.} during preincubation. Preliminary results\textsuperscript{a} have shown that both UDP-GlcNAc and UDP-HexNAcUA are required in the preincubation mixture and that UDP-GlcNAc is required only in the preincubation mixture. Thus it is tempting to speculate that during preincubation some primary acceptor was modified by the addition of 1 or more residues each of N-acetylglucosamine and N-acetylmuramuramic acid so that it was transformed into a suitable secondary acceptor upon which polysaccharide was synthesized as measured by [\textsuperscript{14}C]glucose incorporation. It is interesting to note in Fig. 8 that, although most of the incorporation of [\textsuperscript{14}C]N-acetylmuramuramic acid was dependent upon UDP-Glc, a significant amount of incorporation (about 20\%) occurred during the preincubation period when UDP-Glc was not present. The primary acceptor might be the active component of the S-100 or a tightly bound constituent of the particulate enzyme fraction or both, \textit{i.e.} they might be equivalent. If the modification of the primary acceptor proceeded slowly, it could account for the delay in [\textsuperscript{14}C]glucose incorporation observed as the lag period. If the primary acceptor had the structural composition of a fragment of peptidoglycan, then the rate-limiting reaction might be the introduction of residues, which in the cell wall would be defined as the linkage region between peptidoglycan and the polysaccharide.

The structure of such a linkage region which joins the polysaccharide to peptidoglycan in \textit{M. lysodeikticus} has been established on the basis that the polysaccharide isolated from cell walls lysed with lysozyme alone or in combination with other enzymes which hydrolyse the peptidoglycan, was accompanied by a loss of the linkage region (2). Muramic acid 6-phosphate has been isolated from the purified polysaccharide (2), and the phosphate is very probably a part of the linkage region. Analyses of isolated polysaccharide (2, 9) show a slight excess of glucosamine over and above that necessary to be equimolar with muramic acid. It is possible that the excess glucosamine may also be a part of the linkage region. If this were the case, one might expect the incorporation of 1 or probably not more than 2 residues of N-acetylglucosamine in each strand of polysaccharide newly formed on an acceptor of peptidoglycan. The requirement for UDP-GlcNAc does not appear to be related to \textit{in vitro} synthesis of peptidoglycan, since the synthesis of the polysaccharide was not dependent upon addition of UDP-MurNAc-pentapeptide.

If the entire amount of radioactivity incorporated into product from UDP-Glc[N\textsuperscript{14}C]Ac as shown in Fig. 8 is assumed to represent incorporation into the linkage region, it corresponds to about 1 residue of N-acetylglucosamine for each 15 residues of glucose. In other experiments the ratio was of the order of 1 residue of N-acetylglucosamine for each 20 residues of glucose. These values compare not unfavorably with analytical data on isolated polysaccharide of 1 mole of phosphate for each 10 residues of glucose (2).

\textsuperscript{a} J. S. Anderson, unpublished results.

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