Synthesis of Capsular Polysaccharide (Hyaluronic Acid) by Protoplast Membrane Preparations of Group A Streptococcus*

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Previous reports from this laboratory demonstrated the synthesis of hyaluronic acid by a cell-free preparation obtained from a strain of Group A streptococcus. The enzymic activity which sedimented at 105,000 × g, but not at 10,000 × g, required the addition of uridine diphospho-N-acetylglucosamine, uridine diphosphogluconic acid, and Mg++ for the formation of hyaluronic acid (1–3). Since hyaluronic acid occurs in the capsule of streptococci, this system affords an opportunity for the localization of synthesis of a high molecular weight polymer found external to the cell. The availability of “phage-associated lysin” capable of solubilizing the cell wall of Group A streptococcus (4) facilitated the preparation (5) of protoplast membranes used in this study.

This paper reports the synthesis of hyaluronic acid by protoplast membrane preparations that are relatively free of nucleic acids and cell wall material. A preliminary report of this work has appeared (6). Additional information regarding the properties of the hyaluronic acid-synthesizing system is presented.

EXPERIMENTAL PROCEDURE

UDP-GNAc, UDP-GA, GNAc-1-P and tritiated GNAc-1-P were obtained as described previously (2). UDP-GA (85%) used in Experiment 2 of Table I, was obtained from Sigma Chemical Company. The di- and triphosphates of uridine adenosine, guanosine, cytidine, and inosine were obtained from Sigma Chemical Company and Pabst Laboratories.

Preparation and Purification of UDP-GNAc-H3—UDP-GNAct-H3 was synthesized by the incubation of UDP-GNAct pyrophosphorylase3 at 37° for 255 minutes with the following: GNAc-1-P, 0.55 mM (1.2 × 106 d.p.m. per pmole), UTP, 0.85 mM, NaF, 75 mM; cysteine-HCl, 1.25 mM; MgCl2, 12.5 mM, phosphate, 30 to 40 mM, pH 7.0. The reaction was stopped by boiling after the addition of 1 volume of absolute ethanol. UDP-GNAct-H3 was isolated on Dowex 1-8X-Cl- (200 to 400 mesh, incation after the addition of 1 volume of absolute ethanol. UDP-GNAct, UDP-GA, GNAc-1-P and tritiated GNAc-1-P were obtained from Sigma Chemical Company. The di- and triphosphates of uridine nucleic acids and cell wall material. A preliminary report of this work has appeared (6). Additional information regarding the properties of the hyaluronic acid-synthesizing system is presented.

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1 The UDP-GNAct pyrophosphorylase added was a lyophilized preparation of a 105,000 × g supernatant fraction of sonically disrupted Group A streptococcus (strain A111) obtained as described previously (2).

2 The specific activity of the UDP-GNAct as compared with the starting material, GNAc-1-P-H3, was due to the presence of radioactive contaminants in the GNAc-1-P-H3, demonstrated by paper chromatography.

3 Hexosamine was determined by the Elson Morgan reaction as modified by Boas (9), unless otherwise indicated, after hydrolysis for 14 hours with 4 N HCl at 100°. Dowex 30 treatment was omitted. N-Acetylhexosamine was determined by the Reissig, Strominger, and Leloir (10) modification of the Morgan-Elson reaction, uronic acid by the carbazole method (11), rhamnose by the cysteine-sulfuric acid method (12), nitrogen by a colorimetric micro-Kjeldahl method, phosphate by the Fiske and SubbaRow method (13), and “total” carbohydrate by the method of Dubois et al. (14). The latter determination did not include hexosamine.

4 Protein was determined by a modification of the method of Schneider (15) and RNA and DNA by the orcinol and diphenylamine reactions, respectively, as described by Schneider (16). Ribose and deoxyribose were used as standards.

5 Pancreatic RNase (5 x crystallized) was obtained from the Nutritional Biochemicals Corporation, DNase (1 x crystallized) and crystalline soy bean trypsin inhibitor from Worthington Biochemical Corporation, trypsin (2 x crystallized according to Northrop) from Mann Research Laboratories, and diisopropylfluorophosphate from Eli Lilly and Company. A filtrate of a Streptomyces albus culture was a gift of Dr. H. D. Slade. Bothrops atrox and Naja naja venoms were obtained from Ross Allen's Reptile Institute, Silver Springs, Florida. The venoms were boiled for 15 minutes in 0.03 M sodium acetate, pH 5.9. The inactivation of enzymes that degraded UDP-GA and UDP-GNAct was determined by paper chromatography of the nucleotides after incubation with the boiled enzyme preparation. Phospholipase A was stable to this treatment as determined by an erythrocyte hemolysis assay (17).
antiserum used for the study of "particulate enzyme" was purchased from Difco Laboratories. In experiments with protoplast membranes, rabbit Group A streptococcus antiserum, which was absorbed with Group A streptococci, was prepared by Dr. E. N. Fox. Acid extracts of streptococcal fractions were prepared according to Lancefield (18). Precipitin tests, using Group A streptococcus antiserum, were performed as described by Switt, Wilson, and Lancefield (19). In preliminary experiments, a gift of a preparation of phage associated lysin (4) by Dr. R. M. Krause was used to prepare protoplasts of Group A streptococcus Type 18 (strain A111). The phage-associated lysin used in the experiments detailed here was prepared by infecting 15 liters of an exponentially growing culture of Group C streptococcus (strain 26RP66) in Todd-Hewitt (Difco) broth with sufficient bacteriophage (strain Cl) to cause lysis in 1 hour as determined in separate experiments (4). Thirty five minutes after infection, and before lysis occurred, the culture was cooled by placing it in a 0°C bath and stirring until the temperature had reached 10°C. The infected cells were removed by centrifugation in a continuous flow Servall centrifuge at 4°C. These cells were found to be susceptible to osmotic lysis. They were resuspended to a final volume of 40 ml in 0.05 M phosphate-0.006 M cysteine-0.005 M MgCl₂, pH 7.0. For this purpose 0.5 ml increments of solution were added with vigorous agitation by a rubber policeman until a volume of about 5 ml was reached. The viscosity of the solution was reduced by the addition of 4 mg of DNase. The resulting suspension was centrifuged at 34,800 × g for 30 minutes and the supernatant solution used as a source of phage-associated lysin (4). In Experiment 2 of Table I, aliquots stored at −70°C, were thawed before use and recentrifuged two times at 34,800 × g for 30 minutes to insure removal of protoplast membranes of Group C streptococci.

One unit of phage-associated lysin is defined as the amount required to reduce the optical density of a suspension of Group A streptococcus Type 18 (strain A111) by 1 optical density unit in 1 hour at 37°C when read in tubes (12 × 75 mm) in the Coleman Junior spectrophotometer at a wave length of 650 mm in a suspension containing the following: Na₂HPO₄-KH₂PO₄ (pH 7.0), 0.006 M; cysteine, 0.006 M; Todd-Hewitt (Difco) broth, 2.3%; and strain A111 cells to give an optical density between 0.3 and 0.4. The decrease in optical density was linear for at least 20 minutes when the decrease was about 0.1 in that time. The phage-associated lysin used contained 460 units per mg of protein. The intact protoplasts were sedimented at 6,000 × g for 15 minutes and washed with 1.2 M NaCl containing 20 mM phosphate, pH 7.0. Samples were fixed for electron microscopy by treatment with 3% formaldehyde for 1 hour at 37°C and were subsequently washed with distilled water. Such samples were sprayed on electron microscope grids from a suspension containing the following: phosphotungstate (pH 7.0), 1%; ethanol, 9.5%; Triton X-100, 0.025%; latex balls, 264 μm in diameter.

**RESULTS**

**Chemical and Serological Analyses of Particulate Enzyme Preparation from Sonically Disrupted Streptococci**—A particulate enzyme preparation was prepared for analysis by dialysis against distilled water followed by centrifugation and lyophilization. Qualitative precipitin tests, using Group A streptococcus antiserum (Difco), were positive with acid extracts of the particulate enzyme preparation.

These findings suggested the presence of cell wall material in the particulate enzyme preparation since the serologically reactive polysaccharide of Group A streptococci is localized in the cell wall (20). Consistent with this idea was the finding of nitrogen, 6.7%; hexosamine, 5.6%; rhamnose, 7.3%; and "total" carbohydrate, 9.1%. The above analyses may be compared with the analyses of cell wall preparations by McCarty and Lancefield (21); nitrogen, 7.5%; reducing carbohydrate, 60%; and hexosamine, 20%. Rhamnose has been shown to be a constituent of cell walls of Group A streptococci (20). Electron photomicrographs of this material demonstrated only the presence of a variety of subcellular particles with no identifiable structures present.

**Synthesis of Hyaluronic Acid by Protoplast Membrane Preparations**—Protoplast membranes and the 34,800 × g supernatant fractions were prepared and analyzed (Table I). The chemical analyses for rhamnose, hexosamine, RNA (ribose), and DNA (purine bound deoxyribose) are given in terms of micrograms per mg of protein, whereas the capacity to synthesize hyaluronic acid is expressed as disintegrations per minute per mg of hyaluronic acid per mg of protein (enzyme). The protein of the protoplast membrane represents about 20% of the total protein of the cells. The results in Table I show that the protoplast membranes that have been washed by centrifugation three to six times contain the enzyme(s) responsible for synthesis of hyaluronic acid from UDP-GA and UDP-GNAc-H₂. On a per cell basis, at least 95% of the enzyme(s) reside in the cell membrane and the specific enzymic activity of the membranes is at least 99 times that of the 34,800 × g supernatant fraction. In other experiments, not detailed here, comparison of preparations treated with DNase with those not so treated indicated that DNase did not inactivate the hyaluronic acid-synthesizing system. The effect of RNase was tested in both experiments of Table I. The presence of RNase appeared to result in a loss of activity in Experiment 1; however, this difference was not evident in Experiment 2 and in other experiments in which 100 μg of RNase were included during the synthesis in *vivo* of hyaluronic acid (incubations similar to those described in Table I). Analyses from both experiments show that the addition of RNase did not reduce the quantity of RNA found in the membranes. The results in Table I, Experiment 2b, indicate that sonic die...
Synthesis of hyaluronic acid by protoplast membranes

Experiment 1. Washed cells of strain A111 from 2.8 liters were divided into two portions and each suspended in a final volume of 13 ml containing the following: Na$_2$HPO$_4$-KH$_2$PO$_4$ (pH 7.5), 0.05 M; cysteine, 0.006 M; MgCl$_2$, 3.8 X 10$^{-4}$ M; phage associated lysin, 3000 units (also containing 0.1 mg of DNase). In Experiment 1a, 10 mg of RNase and 1 mg of DNase were added. After an incubation of 10 minutes at 37$^\circ$, the MgCl$_2$ concentration was raised to 0.02 M and the incubation continued for 10 minutes. The suspensions were then chilled and centrifuged at 34,800 X g (average) for 15 minutes. The pellets were resuspended in approximately 12 ml of 0.05 M PO$_4$-0.006 M cysteine, pH 7.5, and centrifuged as before. The process was repeated until the pellet fractions had been washed three times and were finally resuspended in the original volumes in the buffer solutions used for washing the membranes.

Experiment 2. Washed cells of strain A111 from 5.2 liters were divided into two portions and each suspended in a final volume of 20 ml containing the following: Na$_2$HPO$_4$-KH$_2$PO$_4$ (pH 7.5), 0.05 M; cysteine, 0.006 M; MgCl$_2$, 8.7 X 10$^{-4}$ M; phage associated lysin, 10,500 units (also containing 0.05 mg DNase). In Experiment 2a, 10 mg of RNase and 1 mg of DNase were added. After incubation for 15 minutes at 37$^\circ$, the MgCl$_2$ concentration was increased to 0.02 M and incubation continued for 10 minutes. The suspensions were chilled and centrifuged at 34,800 X g for 15 minutes. The pellets were resuspended in approximately 30 ml of 0.05 M Na$_2$HPO$_4$-KH$_2$PO$_4$-0.006 M cysteine, pH 7.5 and centrifuged as before. The pellets were resuspended, centrifuged at 3,000 X g (average) for 5 minutes and these pellets discarded. The supernatant suspensions were centrifuged at 34,800 X g for 15 minutes and the pellet fractions resuspended in buffer. These 34,800 X g pellets were washed by repeated centrifugation at 34,800 X g for 15 minutes four additional times (a total of six washings). The protoplast membranes were finally resuspended to the original volumes in the buffer solutions used for washing the membranes.

A sample of 0.75 ml of each enzyme fraction, containing the amount of protein indicated, was added to each tube in a final volume of 1.5 ml containing the following: Na$_2$HPO$_4$-KH$_2$PO$_4$ (pH 7.5), 0.05 M; cysteine, 0.004 M; MgC$_2$, 0.033 M; UDP-GA, 1.0 mM; UDP-GNAc-H$_3$ (1.5 X 10$^6$ d.p.m.), 1.0 mM. Samples were incubated 2 hours at 37$^\circ$. Unincubated controls were included for each enzyme treatment.

The analyses on all supernatant fractions have been corrected for the amount of rhamnose, hexosamine, RNA, DNA, and protein added with lysin, DNase, or RNase.

The entire supernatant from a preparation containing from 1.2 to 2.6 mg of protein was analyzed.

* The analyses on all supernatant fractions have been corrected for the amount of rhamnose, hexosamine, RNA, DNA, and protein added with lysin, DNase, or RNase.

† The entire supernatant from a preparation containing from 1.2 to 2.6 mg of protein was analyzed.

### Table I

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Treatment</th>
<th>Enzyme fraction</th>
<th>Specific activity of HA synthesizing system*</th>
<th>Chemical composition of fractions†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>HA/mg protein</td>
<td>Rhamnose</td>
</tr>
<tr>
<td>1a</td>
<td>Lysin + RNase + DNase</td>
<td>1) Supernatant of lysate centrifuged at 34,800 X g for 15 min</td>
<td>6.0</td>
<td>210</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2) Protoplast membranes washed 3 times</td>
<td>2.7</td>
<td>21,900</td>
</tr>
<tr>
<td>1b</td>
<td>Lysin + DNase</td>
<td>1) Supernatant of lysate centrifuged at 34,800 X g for 15 min</td>
<td>7.4</td>
<td>330</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2) Protoplast membranes washed 3 times</td>
<td>1.8</td>
<td>33,900</td>
</tr>
<tr>
<td>2a</td>
<td>Lysin + RNase + DNase</td>
<td>1) Supernatant of lysate centrifuged at 34,800 X g for 15 min</td>
<td>8.0</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2) Protoplast membranes washed 6 times</td>
<td>2.2</td>
<td>11,600</td>
</tr>
<tr>
<td>2b</td>
<td>Lysin + DNase</td>
<td>1) Supernatant of lysate centrifuged at 34,800 X g for 15 min</td>
<td>8.7</td>
<td>53</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2) Protoplast membranes washed 6 times</td>
<td>1.9</td>
<td>11,900</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3) Protoplast membranes washed 6 times and subjected to sonic disruption for 30 min at 9 kc.</td>
<td>1.9</td>
<td>3,700</td>
</tr>
</tbody>
</table>

* Corrected to 10 mg of carrier hyaluronic acid; 11.8 mg were added to each tube.
† The analyses on all supernatant fractions have been corrected for the amount of rhamnose, hexosamine, RNA, DNA, and protein added with lysin, DNase, or RNase.
‡ The entire supernatant from a preparation containing from 1.2 to 2.6 mg of protein was analyzed.

ruption of well washed protoplast membranes results in marked loss of activity. The inactivation of enzyme(s) of washed membranes may result from greater lability of the more purified material. Centrifugation of these sonically disrupted protoplast membranes for 5 hours at 105,400 X g (maximum) revealed that for 30 minutes of lysates of washed protoplasts increased their enzyme activity by a factor of two. Therefore, all the cells did not lyse immediately even though the cell walls had been almost completely removed at this point.
all of the remaining activity was still sedimentable, although 13% of the protein was in the supernatant fluid (conditions for testing the supernatant and pellet fractions were similar to those described in Table I).

The chemical analyses of the supernatant fluid and membrane fractions for cell wall constituents of Group A streptococci (rhamnose and hexosamine) show that, on a per cell basis, about 2% of the rhamnose and 2% of the hexosamine are still present in the washed protoplast membranes. This amount was not reduced by exposure to lysin for longer periods of time (experiments not detailed here). Qualitative precipitin tests, using Group A streptococcus antiserum, were positive with acid extracts of the washed protoplast membranes of Table I, Experiment 2a. When the antiserum was first reacted with whole Group A streptococci, to remove the Group A (cell wall) reactive antibody, the antiserum was no longer reactive with the same acid extracts of the washed protoplast membranes.

Although no DNA was found in the hot trichloroacetic acid extracts of membranes, as much as 2% of the total cellular DNA would not have been detected. The RNA found in the membranes (Table I, Experiment 2b) represents 1.3% of that found in the supernatant fraction on a per cell basis.

Photographs 1, 2, and 3 of Fig. 1 are electron photomicrographs of protoplast membranes obtained by lysis of washed protoplasts in hypotonic buffer and centrifugation at 15,000 × g for 30 minutes. Considerable RNA (33 μg of ribose per mg of protein) was still present. Photograph 4 represents these membranes treated with additional DNase and RNase and containing little RNA (3 μg of ribose per mg of protein). The protoplast membranes appear to be almost intact, flattened spheres. The

![Fig. 1. Electron photomicrographs of protoplast membranes. The latex balls (light appearing with respect to phosphotungstate in photographs 1 and 4; dark appearing in photograph 2) are 264 μm in diameter.](http://www.jbc.org/)

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variability of size of the protoplast membranes in photograph 3 has been previously observed (5). Detailed inspection of a number of fields revealed only structures that could be identified as protoplast membranes.

**Experiment to Detect Primer**—Previous studies with washed particulate enzyme from sonically disrupted cells showed that hyaluronic acid was synthesized without the addition of primer (2). Since almost all other polysaccharide synthesizing systems exhibit a primer requirement, it seemed of importance to determine whether or not primer might originate from the enzyme preparation. In view of the fact that the requisite amounts might be below those detectable by analytical techniques, a C\(^{14}\)labeled particulate enzyme preparation was obtained from cells grown in the presence of acetate-\(^{14}\)C. Under these conditions, any hyaluronic acid attached to the enzyme might be expected to be labeled in the acetyl group (22). As indicated in Table II, this enzyme preparation was reacted with inactive UDP-GA and UDP-GNAc. Simultaneously, the extent of synthesis in vitro was measured by using UDP-GA and UDP-GNAc-H\(^{3}\) (Table II). The results indicate that, during the 2-hour incubation period, 180 \(\mu\)g of hyaluronic acid were synthesized (Tube 6), of which 2.2 \(\mu\)g may have originated from the enzyme (Tube 4 minus Tube 2). The amounts are too small for accurate estimation; however, it is apparent that at most about 1% of the hyaluronic acid may have been derived from the enzyme preparation. Whether this represented actual hyaluronic acid (or oligosaccharides) or simply radioactive contamination cannot be ascertained.

The specific enzymic activity of the particulate enzyme, calculated from the data of Table II and corrected as indicated in Table I, is 12,900 d.p.m. per mg of hyaluronic acid per mg of enzyme in 2 hours at 37\(^\circ\). Analysis of the hot trichloroacetic acid was discarded and the enzyme obtained by centrifugation for 60 minutes at 105,400 \(\times\) g. The 105,400 \(\times\) g pellet was suspended in the original volume (25 ml) in the same buffer solution.

**Table II**

<table>
<thead>
<tr>
<th>Tube No.</th>
<th>Additions</th>
<th>Incubation</th>
<th>Specific activity of hyaluronic acid</th>
<th>Hyaluronic acid from particulate enzyme</th>
<th>Net synthesis of hyaluronic acid in vitro</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>None</td>
<td>60</td>
<td>d.p.m. /mg</td>
<td>ps</td>
<td>ps</td>
</tr>
<tr>
<td>2</td>
<td>None</td>
<td>77</td>
<td>0</td>
<td>5.3</td>
<td>5.3</td>
</tr>
<tr>
<td>3</td>
<td>UDP-GA +</td>
<td>0</td>
<td>110</td>
<td>7.5</td>
<td>(180)</td>
</tr>
<tr>
<td>4</td>
<td>UDP-GA +</td>
<td>2</td>
<td>UDP-GNAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>UDP-GA +</td>
<td>0</td>
<td>UDP-GNAC-H(^{3})</td>
<td>(1.53 (\times) 10(^4) d.p.m.)</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>UDP-GA +</td>
<td>2</td>
<td>UDP-GNAC-H(^{3})</td>
<td>(1.53 (\times) 10(^4) d.p.m.)</td>
<td>180†</td>
</tr>
</tbody>
</table>

* The micrograms of hyaluronic acid “transferred” from the particulate enzyme were calculated as follows:

\[ \text{Specific activity of hyaluronic acid-C}^{14} (\text{d.p.m./mg}) \times \text{mg of carrier hyaluronic acid} \]

\[ \text{Specific activity of capsular hyaluronic acid-C}^{14} \text{ synthesized in vivo (d.p.m./\(\mu\)g)} \]

The capsular hyaluronic acid-C\(^{14}\) was isolated from the washings of the cells without the addition of carrier and had a specific activity of 1.4 \(\times\) 10\(^4\) d.p.m./mg.

† The micrograms of hyaluronic acid synthesized during the 2-hour incubation were calculated as follows:

\[ \text{Total activity of UDP-GNAC-H}^{3} \text{ added} = \frac{(48,300) (9.55) (1.57) (379)}{1.53 \times 10^{6}} \]

Group A streptococci (strain A111) were grown in the presence of 1 mc of sodium acetate-\(^{14}\)C (13.8 mg), washed, and subjected to sonic disruption in 0.05 \(\times\) phosphate-0.004 \(\times\) cysteine-HCl (pH 7.5) for 60 minutes at 0\(^\circ\) in a Raytheon 9 kc. sonic oscillator. Following centrifugation at 10,000 \(\times\) g for 10 minutes, the pellet was discarded and the enzyme obtained by centrifugation for 90 minutes at 105,400 \(\times\) g (average). The 105,400 \(\times\) g pellet was suspended in the original volume (25 ml) in the same buffer solution and recentrifuged for 60 minutes at 105,400 \(\times\) g. The pellet was washed two more times and finally resuspended in 0.05 \(\times\) phosphate-0.004 \(\times\) cysteine-HCl. Particulate enzyme, containing 3.57 mg of protein and 1.7 \(\times\) 10\(^6\) d.p.m. of C\(^{14}\), was added to each tube in a final volume of 1.5 ml containing the following: Na\(^{+}\)HPO\(_{4}\)-KH\(_{2}\)PO\(_{4}\) (pH 7.5), 0.05 \(\times\); cysteine, 0.004 \(\times\); MgCl\(_{2}\), 0.033 \(\times\). Where indicated the following were added: UDP-GA, 1.0 \(\mu\)m; UDP-GNAc, 1.0 \(\mu\)m. Samples were incubated at 37\(^\circ\). Hyaluronic acid (9.56 mg) was added as carrier at the end of the incubation period and was purified (2, 3). All results reported in the table are the average of independent duplicate samples carried through the entire procedure.
Although the hyaluronic acid purification involves a number of contamination. The amount of 04 that is present in the carrier hyaluronic acid after Cl* found in the reisolated carrier hyaluronic acid could be due to particulate enzyme isolated from cells grown on acetate-L-W. The possibilities must be considered that part of the treatments did reduce somewhat the amount of RNA in the thesis occurred (26, 27).

Charide by muscle phosphorylase has also been demonstrated in composed of alternating residues of N-acetylglucosamine and glucuronic acid. Recently, the synthesis de novo of polysaccharide phosphorylase (26, 27).

Although the initial studies of the synthesis in vitro of deoxy-ribonucleic acid showed that a deoxyribonucleic acid “primer” was necessary for net synthesis of DNA (24) more recent experiments indicate that a polymer of deoxyadenylate and deoxythymidylate can be synthesized by the same polymerase preparation without the addition of primer, although a lag of 3 to 4 hours occurs. In addition, the “unprimed” synthesis gives rise to a macromolecule containing deoxyadenylate and deoxythymidylate “in perfectly alternating sequence” (23). Such a polymer is similar to hyaluronic acid in the sense that hyaluronic acid is composed of alternating residues of N-acetylglucosamine and glucuronic acid. Recently, the synthesis de novo of polysaccharide by muscle phosphorylase has also been demonstrated in the absence of primer and was found to involve a lag before synthesis occurred (26, 27).

Protoplast membranes containing little cell wall material and DNA may be obtained from Group A streptococcus by treatment with a phage-associated lysin (4, 5, 28). The data of Table I are in general agreement with these findings. The definition of a “pure protoplast membrane,” at the present time, can only be considered from an operational point of view. The results of Table I demonstrate that the cell wall constituents, rhamnose and hexosamine, could be detected in the membranes although at much lower levels than in the supernant fractions. In addition, acid extracts of the membranes reacted with Group A streptococcus antiserum indicating the presence of a serologically reactive cell wall component. A small amount of RNA and possibly DNA were also present. The cell wall constituents in the membrane fraction were reduced to a constant level. This level was not changed by increasing the amount of phage-associated lysin, removing the material sedimenting at 3,000 x g in 5 minutes (intact protoplasts?), and increasing the number of washings (Table I, compare Experiments 1 and 2). These latter treatments did reduce somewhat the amount of RNA in the membranes. The possibilities must be considered that part of the cell wall rhamnose and hexosamine are not susceptible to the enzymic action of the lysin (analogous to limit dextrins found on incubation of phosphorylase with glycozyme) or that the rhamnose and hexosamine may be constituents of the cell membrane. In this connection the analysis of the protoplast membranes of Micrococcus lysodeikticus prepared with lysozyme is of some interest. These protoplast membranes contain 2.5 to 3% hexosamine (as glucosamine) and 17.5 to 19.7% total sugar (as mannose), whereas the cell walls of this organism contain 16 to 23% hexosamine and 7.5 to 10.4% total sugar (as glucose) (20) (see also review (30)).

The amount of RNA present in the washed particulate enzyme (4.6 μg of ribose per mg of protein) and in the washed protoplast membranes (Table I, Experiment 2; 4.3 to 5.1 μg of ribose per mg protein) was quite low. Ribonuclease treatment did not affect the quantity found. The most important consideration with respect to RNA and DNA, from a bioisotopic point of view, is that RNase and DNase did not affect the activity of the hyaluronic acid-synthesizing system. Other systems in which RNA and DNA participate are sensitive to the particular nuclease (24, 31–35). The participation of DNA and RNA in the biosynthesis of heteropolysaccharides via a template mechanism has been suggested (36–38). The present data do not support this suggestion although they do not completely negate this possibility.

A comparison of the specific enzyme activities of the particulate enzyme obtained from sonically disrupted cells (12,900 d.p.m. per mg of hyaluronic acid per mg of protein, calculated from Table II) and from protoplast membranes (see directly comparable figures in Table I) shows them to be quite similar.

Repeated attempts to solubilize the hyaluronic acid-synthesizing system using phospholipase A have led neither to solubilization nor inactivation despite the fact that the membranes of Group A streptococcus have been reported to contain about 25% lipid with a high phosphorus content (39). The lipid of protoplast membranes of Micrococcus lysodeikticus is about 80% phospholipid (29), and phospholipase A has been used successfully to solubilize α-glycero-phosphophosphate dehydrogenase (40) and choline dehydrogenase (41). The hyaluronic acid-synthesizing system of the membranes may not be bound to a compound sensitive to phospholipase A or may be located in such a position as to be inaccessible to phospholipase A action. Difficulties in solubilizing the enzymic activity by physical or chemical means may indicate a requirement for an organized structure.

Summary

Attempts have been made to evaluate the amount of “primer” bound to the particulate hyaluronic acid-synthesizing system obtained from sonically disrupted Group A streptococci grown on acetate-1-C4. No more than 1% of the hyaluronic acid formed could have been derived from primer.

At least 95% of the hyaluronic acid-synthesizing activity of streptococci was recovered in washed protoplast membrane preparations. The hyaluronic acid-synthesizing system of these preparations had a specific enzymic activity similar to the particulate enzyme(s) obtained from sonically disrupted cells and was not inactivated by ribonuclease or deoxyribonuclease despite the fact that after such treatment little ribonuclease and deoxyribonuclease could be detected in the membranes. Chemical analyses of the protoplast membrane preparations are presented.
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Synthesis of Capsular Polysaccharide (Hyaluronic Acid) by Protoplast Membrane Preparations of Group A Streptococcus
Alvin Markovitz and Albert Dorfman


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