Solubilization and Partial Purification of Hyaluronate Synthetase from Oligodendroglioma Cells*

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Hyaluronate synthetase was solubilized with digitonin from crude membranes of mouse oligodendroglioma cells. Detergent extraction was carried out in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid-buffered saline with an optimal digitonin to protein ratio (w/w) of 0.7–0.8. The solubilized synthetase was partially purified approximately 230-fold by gel filtration and ion-exchange chromatography. The solubilized enzyme displayed similar properties to membrane-bound enzyme: (a) it synthesized high molecular weight hyaluronate which eluted in the void volume of a Sepharose CL-2B column; (b) the apparent $K_m$ values obtained for UDP-GlcUA and UDP-GlcNAc were 50 and 100 mM, respectively; and (c) treatment of intact cells with hyaluronidase prior to extraction with digitonin resulted in a 3-fold increase in solubilized synthetase activity. Furthermore, gel filtration chromatography of the solubilized hyaluronidase-treated synthetase complex showed that it was smaller than the solubilized untreated synthetase complex, due to shorter nascent-bound hyaluronate. The solubilized synthetase was shown to be associated with hyaluronate in the form of a complex. Both hyaluronidase-treated and -untreated synthetase-hyaluronate complexes after solubilization were adsorbed by an affinity matrix using the hyaluronate binding domain of rat chondrosarcoma proteoglycan as ligand. This solubilized active enzyme preparation should allow the identification and characterization of the components of the hyaluronate-synthetase complex.

Hyaluronate is a linear chain glycosaminoglycan with a repeating unit structure $\{(1\rightarrow4)-\beta-D$-glucuronosyl-(1$\rightarrow3$)-N-acetylglucosaminyl$\}$. The molecular weight of the chain is variable but often exceeds $10^6$ daltons. Hyaluronate is widely distributed in both vertebrate and invertebrate connective tissues and is also found as a component of the cell wall of group A streptococci.

Most of the early knowledge of hyaluronate synthesis is derived from studies using streptococci, mainly because of the ease in producing this organism in copious quantity. The exclusive synthesis of only one glycosaminoglycan, and the high specific activity of the bacterial enzyme preparation (1–3). Even though hyaluronate from both procaryotic and eucaryotic cells is structurally similar, the biosynthetic mechanism may be different. In streptococcus, the mechanism of synthesis has been shown to be by alternate transfer of monosaccharides from UDP-glucuronic acid and UDP-$N$-acetylglucosamine to the nascent chains (2, 3). Although it has been proposed that a similar mechanism of synthesis may exist in a eucaryotic cell line, differentiated teratocarcinoma (4), available evidence does not eliminate the participation of a disaccharide unit, possibly via lipid intermediates (5, 6) as in glycoprotein synthesis. In fact, based on an explicit kinetic model of hyaluronate synthetase from oligodendroglioma cells (7), evidence has been obtained which suggests such a mechanism. In streptococcus, sugars are added to the nonreducing end of the growing hyaluronate chain (3) while the acceptor terminus has not been identified in eucaryotic cells (4, 7).

Prehm (8) has presented evidence that the growing hyaluronate chain is bound covalently to the enzyme through the nucleotide sugar in tetratocarcinoma cells, but this is contradictory to previous work (2, 3) and has not been confirmed by others (7, 9). Another enigma concerning the biosynthesis of hyaluronate involves the subcellular site of synthesis of this large polysaccharide. We have previously shown that the hyaluronate synthetase is localized at the inner surface of the plasma membrane in the glioma cells (10). The site of synthesis of hyaluronate is thus different from that of other glycosaminoglycans in eucaryotic cells but similar to the proteolytic membrane site in bacteria. This unique subcellular localization helps explain some of the unusual features of hyaluronate synthesis, but the significance to the cell is not readily apparent. In order to resolve these important questions, as well as elucidate the role of primer molecules and the direction of synthesis, and to advance our knowledge of the hyaluronate synthesizing system, it is important to solubilize the enzyme system. Recent efforts have been successful with the cellulose synthetase from Acetobacter xylinum (11) and the hyaluronate synthetase from Streptococcus (12, 13).

In this report we present evidence on the first successful solubilization of functional hyaluronate synthetase from a eucaryotic cell line, mouse oligodendroglioma. Furthermore, the synthetase has been partially purified through gel filtration and ion-exchange chromatography.

**EXPERIMENTAL PROCEDURES**

**Materials**—The mouse oligodendroglioma cell line (strain G26-24) was generously provided by Dr. G. Dawon (University of Chicago). The strain was originally isolated by Sundarraj et al. (14) from an immature cell tumor induced by methylcholanthrene treatment of C57BL/6J inbred mice (15). The cells were grown as described previously (7, 10).

Human umbilical cord hyaluronate was a gift from Dr. Martin B. Mathews (University of Chicago). Heparin (157 U.S.P. units/mg) was from Riker Laboratories. Bovine $\gamma$-globulin, protein assay dye reagent concentration, and hydroxyapatite (enzyme grade) were obtained from Bio-Rad. Bovine testicular hyaluronidase (15,000–20,000 I.U./mg) was purchased from Leo, Helsingborg, Sweden. Sephadex G-50 (superfine), Sepharose CL-6B, Sepharose CL-4B, and Sepharose CL-
2B were obtained from Pharmacia LKB Biotechnology Inc. Dithiothreitol was obtained from Bethesda Research Laboratories. The following materials were purchased from Sigma: octyl-β-D-glucopyranoside, Chaps,1 sodium deoxycholate, digitonin (80% pure), Triton X-100, uridine diphosphoglucuronic acid, and uridine diphospho-N-acetylglucosamine-[U-14C]glucuronic acid (specific activity 25 mCi/mmol) was purchased from ICN Radiochemicals, Amersham Corp. supplied aqueous scintillation mixture.

Preparation of Crude Membrane—Suspended cells were rinsed by hand inversion in calcium and magnesium-free Hank's balanced salt solution, pelleted by centrifugation at 300 × g for 10 min, cooled on ice to 0-4°C, then pelleted and washed twice with 0.1 M NaCl containing 10 mM Hepes and 0.5 mM dithiothreitol, pH 7.1. Washed cells were resuspended under hypotonic conditions (10 mM Hepes, 0.5 mM dithiothreitol, pH 7.4) and allowed to swell for 1 h on ice. After this treatment, cells were homogenized with 10 strokes in the Dounce homogenizer. An equal volume of 20% sucrose was immediately added to stabilize the subcellular organelles (10, 17). The homogenate was centrifuged at 1,000 × g for 10 min, and the resulting supernatant was centrifuged at 20,000 × g for 20 min. In some experiments, cell layers were rinsed three times with Hank's balanced salt solution (Ca2+- and Mg2+-free) prior to incubation with testicular hyaluronidase (20 IU/ml) in 6 ml of the same buffer for 20 min at 37°C in the presence of 10% CO2 (10). The cell layers were then rinsed thoroughly many times with ice-cold Hank's balanced salt solution (Ca2+ and Mg2+-free) before harvest of cells and isolation of crude membranes as described above.

Achilles of Hyaluronate Synthetase—Hyaluronate synthetase was assayed as described previously (10). A standard assay (unless stated otherwise) contained the following components in a final volume of 0.1 ml: 25 mM Hepes-NaOH, pH 7.1, 5 mM dithiothreitol, 15 mM MgCl2, 1 mM UDP-GlcNAc, 0.05 mM UDP-GlcUA, 0.1-2.5 μCi of UDP-[14C]GlcUA, and 10-200 μg of membrane protein. For the assay of solubilized enzyme preparations (which include a high speed supernatant fraction of detergent extracts as well as fractions collected from Sepharose CL-6B, Sepharose CL-4B, or hydroxyapatite columns) the concentration of UDP-GlcNAc used was 0.1 mM instead of 100 M.

Detergent Extraction—Detergents were suspended or dissolved in Hepes-buffered saline (50 mM Hepes-NaOH, pH 7.1, 200 mM NaCl) containing 15 mM MgCl2, 10 mM dithiothreitol, and 10% glycerol. Crude membranes (5-20 mg/ml of protein) prepared as described above, were resuspended by gentle homogenization in Hepes-buffered saline and mixed with the appropriate detergent. The membranes were then extracted at 4°C for 1 h with gentle stirring. Each extract was subjected to ultracentrifugation for 1 h at 186,000 × g to give a high speed pellet and supernatant fraction. The pellet was resuspended to its original volume in extraction buffer and detergent. For comparison with membrane-bound hyaluronate synthetase activity (40% of the total membrane-bound proteins, while 40% of the crude membranes were assayed for activity and protein content. The assay is compatible with sodium phosphate buffer, and phosphate concentration as high as 300 mM does not interfere with enzyme activity.

Synthesis of Proteoglycan-Sepharose 4B—Rat chondrosarcoma proteoglycan was isolated as described (26). The 80-kDa hyaluronate-binding domain of the proteoglycan was obtained by clostripain digestion and purified by gel filtration under associative conditions (27). It was digested with hyaluronidase and again purified by gel filtration under dissociative conditions. The polypeptide obtained was coupled to CNBr-activated Sepharose 4B in borate buffer.

Hyaluronidase Digestion—[14C]Glucuronic acid-labeled or unlabeled hyaluronic acid was digested with testicular hyaluronidase overnight at room temperature in 0.1 M sodium acetate buffer, pH 5.0, and 0.15 mM NaCl.

Kinetics of Hyaluronate Synthetase—Steady state kinetic data were processed on a DEC-20 computer with the Basic ENZYKIN program as described previously (7).

Protein Determination—Protein determination of membrane bound proteins were carried out using the Lowry method as modified by Markwell et al. (18). Solubilized proteins from detergent extracts and fractions collected from Sepharose CL-6B, Sepharose CL-4B, or hydroxyapatite columns were assayed for hyaluronate synthetase activity under standard assay conditions (see "Experimental Procedures"). In initial solubilization experiments, a range of detergent to protein ratios was used. Each extract was then centrifuged and soluble components (186,000 × g supernatant) were assayed for hyaluronate synthetase activity under standard assay conditions (see "Experimental Procedures").

These activity levels were compared to activity measured in the resuspended high speed pellet and original crude membrane. A larger amount of membrane-bound proteins were solubilized using a high detergent-to-protein ratio. However, this also resulted in inactivation of the hyaluronate synthetase (Fig. 1). At high (11.9 mg/ml) protein concentration an optimum digitonin-to-protein ratio is observed at approximately 0.7. At lower (7.9 mg/ml) protein concentration this ratio shifted to 1.0. Since solubilization only occurs at or near the critical micelle concentration for most detergents, this discrepancy may be due to lowering of the digitonin concentration below the value needed for solubilization at a particular digitonin-to-protein ratio when the protein concentration is low. With regard to hyaluronate synthetase, lower protein concentrations were not studied because of the difficulties in accurately measuring the low level of solubilized activity obtained. In no case was stimulation of activity observed in crude membranes using low (<0.1%) concentration of digitonin.

Under optimal conditions, digitonin solubilized about 30-40% of the total membrane-bound proteins, while 40% of the

1 The abbreviations used are: Chaps, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; SDS, sodium dodecyl sulfate; Hepes, 4-(2-hydroxyethyl)-1-piperazineneethanesulfonic acid; PAPs, adenosine 3'-phosphate 5'-phosphosulfate.
Solubilization of hyaluronate synthetase activity by digitonin. Crude membranes were prepared in Hepes-buffered saline as described under "Experimental Procedures." Aliquots were mixed with different amounts of digitonin which were previously suspended in the same Hepes-buffered saline by sonication. Each extract was stirred gently for 1 h at 4 °C and then centrifuged at 186,000 × g for 1 h. Each supernatant fraction was then assayed for hyaluronate synthetase activity. The concentrations of protein used were 7.9 mg/ml (●), 9.4 mg/ml (□), and 11.85 mg/ml (△).

**TABLE 1**

| Enzyme fraction | Total protein (mg) | Total activity (pmol h⁻¹) | Specific activity (pmol h⁻¹ mg⁻¹) |
|-----------------|--------------------|---------------------------|---------------------------------|
| Untreated control |                    |                           |                                 |
| Crude membranes  | 32.6 (100)         | 3,350 (100)               | 102 (100)                       |
| 186,000 × g pellet | 16.6 (51)         | 150 (5)                   | 9 (9)                           |
| 186,000 × g supernatant | 13.7 (42) | 1,510 (45)               | 110 (108)                       |
| Hyaluronidase-treated |                |                           |                                 |
| Crude membranes  | 30.8 (100)       | 11,430 (100)              | 371 (100)                       |
| 186,000 × g pellet | 17.0 (55)        | 910 (8)                   | 74 (15)                         |
| 186,000 × g supernatant | 11.7 (38) | 4,120 (36)               | 352 (95)                        |

*Parentheses indicate relative values in percentage.

Total activity and 80–110% of the specific activity of hyaluronate synthetase were recovered in the soluble supernatant, when compared to the activity in the original crude membrane (Table 1). Less than 10% of the total activity remained in the extracted pellet. Since total recovery of activity was only 50% in the extracted pellet and supernatant fractions combined, the possibility exists that some enzyme proteins were inactivated during solubilization. Increasing the amount of glycerol in the extraction buffer from 10 to 40% did not result in greater recovery of activity in the supernatant fraction. Both MgCl₂ and dithiothreitol were also found to be essential for good recovery of activity (data not presented), and therefore used routinely. Since the supernatant was assayed directly, the level of digitonin in the assay reaction mixture was high (0.1–0.5%). However, it did not seem to interfere with activity because removal of most digitonin by dialysis for 36 h or dilution of the digitonin extract with buffer did not enhance the specific activity.

In a previous study of hyaluronate synthetase in these cells, Philipson et al. (7) found that treatment of intact cells with the nonpenetrating agent hyaluronidase for 20 min at 37 °C resulted in a 3–4-fold increase in enzyme activity in the washed crude membranes. This effect of hyaluronidase was confirmed in the present study and the hyaluronidase-treated membranes used as a source for solubilizing the synthetase. Treatment of intact cells with hyaluronidase prior to extraction with digitonin resulted in about a 3-fold increase in enzyme activity (Table I). The efficiency of the solubilization procedure in terms of total and specific activities, however,
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FIG. 4. Elution profile of hyaluronate synthetase on Sepharose CL-4B. Digitonin extract of crude membranes from hyaluronidase-treated cells (13.8 mg protein) (a) and from untreated cells (17.1 mg protein) (b) were applied to a column (1 x 120 cm) equilibrated in 50 mM sodium phosphate, pH 7.1, containing 50 mM NaCl, 5 mM MgCl2, 5 mM dithiothreitol, 0.1% digitonin, and 10% glycerol (buffer A) and eluted with the same buffer. Fractions (2.0 ml) were collected at a flow rate of 8 ml h⁻¹. Aliquots (360 μl) from each fraction were assayed for hyaluronate synthetic activity in a final volume of 400 μl under standard assay conditions (see "Experimental Procedures"). The profiles shown are enzyme activity (▽) and protein (●).

FIG. 5. Elution profile of hyaluronate synthetase on Sepharose CL-4B. Digitonin extract of crude membranes from untreated cells (25.7 mg of protein) was applied to a column (1 x 120 cm) equilibrated in buffer A. The elution conditions were as in Fig. 4. Aliquots (460 μl) from each fraction were assayed for activity. The profiles shown are enzyme activity (▽) and protein (●).

was similar for both treated and untreated membranes.

Other detergents were also tested for their ability to solubilize hyaluronate synthetase. These include Chaps, octyl-β-D-glucopyranoside, sodium deoxycholate, and Triton X-100 over a range of detergent to protein ratios. Little (<5%) or no activity was found in either the high-speed supernatant or the resuspended pellet with any of these detergents. In no case could activity be recovered from the supernatant after removal of the detergent by dialysis. Thus, it appears that these detergents failed to solubilize the enzyme in an active form.

Properties of Digitonin Solubilized Activity—Crude membranes were prepared and extracted with digitonin under the optimal conditions just described. The 186,000 x g supernatant fraction obtained was assayed for hyaluronate synthetase activity under various conditions, including a range of incubation times and protein concentrations. The solubilized activity increased linearly with incubation up to 3 h, and with protein from 0.1 to 1.0 mg/ml. The activity was stable for 24 h at 4 °C in 10% glycerol (greater than 90% of activity was recovered) and then decreased rapidly; higher levels of glycerol did not increase stability over time. The solubilized activity from hyaluronidase-treated, washed crude membranes also showed similar behavior in stability.

The nature of the product synthesized by the solubilized system was also assessed. The [14C]glucuronic acid labeled product produced by the soluble hyaluronate synthetase was largely eluted in the void volume of a Sepharose CL-2B column (Fig. 2), indicating a hydrodynamic size greater than 10⁷ daltons. The size of these products was similar to hyaluronic acid synthesized by intact membranes (7). The hyaluronate product was collected, dialyzed against H2O, lyophilized, and digested with testicular hyaluronidase and the digest chromatographed on the same Sepharose CL-2B column. All of the radioactivity was sensitive to hyaluronidase treatment and was eluted at the excluded volume of the column (Fig. 2), demonstrating that [14C]glucuronic acid was largely incorporated into high molecular weight hyaluronate by the solubilized synthetase activity. In our previous studies (10) of the oligodendrogliala cells, streptomycetes hyaluronidase had been used to show that 90% of the [14C] glucuronic acid label taken up by the cells was incorporated...
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The values for A and C were obtained from experiments described under the legends of Figs. 4a, 5, and 6. The values for B were obtained from a separate experiment.

| Fraction         | Volume | Protein | Total protein | Activity | Total activity | Specific activity |
|------------------|--------|---------|---------------|----------|----------------|------------------|
|                  | ml     | mg ml⁻¹ | mg            | pmol h⁻¹| pmol h⁻¹ mg⁻¹ | pmol h⁻¹ mg⁻¹    |
| A. Hyaluronidase-treated |        |         |               |          |                |                  |
| 186,000 x g supernatant | 2.2    | 6.27    | 13.80         | 1,644   | 3,660 (100)   | 265 (1)          |
| Sepharose CL-6B (fractions 20–28) | 18      | 0.14    | 2.67          | 143     | 2,580 (71)    | 965 (3.7)        |
| Hydroxylapatite (40 mM + 80 mM fractions) | 6     | 0.056   | 0.335         | 229     | 2,370 (38)    | 4,100 (15.5)     |
| B. Untreated |        |         |               |          |                |                  |
| 186,000 x g supernatant | 2.8    | 5.07    | 14.20         | 333     | 930 (100)     | 66 (1)           |
| Sepharose CL-6B (fractions 17–21) | 10      | 0.057   | 0.57          | 60      | 600 (64)      | 1,040 (15.8)     |
| C. Untreated |        |         |               |          |                |                  |
| 186,000 x g supernatant | 3      | 8.58    | 25.74         | 744     | 2,250 (100)   | 87 (1)           |
| Sepharose CL-4B (fractions 18–22) | 10      | 0.0085  | 0.085         | 80.6    | 810 (36)      | 9,483 (109)      |
| Hydroxylapatite (40 mM + 80 mM) | 2      | 0.011   | 0.022         | 217     | 430 (19)      | 19,700 (226)     |

* Parentheses after total activity represent percentages.

* Parentheses after specific activity indicate the relative -fold purification.

into high molecular weight hyaluronic acid. This result was comparable with those obtained using testicular hyaluronidase.

Kinetics of Solubilized Hyaluronate Synthetase—The effects of hyaluronidase treatment on the apparent steady-state kinetics of the solubilized synthetase were examined with a 4 x 4 matrix of substrates. The appropriate ranges were found to be 10–100 µM for UDP-GlcUA, and 20–200 µM for UDP-GlcNAc. Higher concentrations of either substrate resulted in inhibition of activity. Double-reciprocal plots of 1/V₀ versus 1/substrate concentration gave a family of parallel lines for solubilized activity from control membranes and a family of intersecting lines for solubilized activity from hyaluronidase-treated membranes (Fig. 3). The apparent Kₘ values obtained were 50 µM for UDP-GlcUA and 100 µM for UDP-GlcNAc. These values were similar to those found previously for the membrane-bound system (10) and suggest that the solubilized enzyme displays regulatory properties similar to the membrane form of enzyme.

Purification of Hyaluronate Synthetase—Once solubilization of a functional synthetase was confirmed, further purification was attempted. Since hyaluronate synthetase is bound to nascent hyaluronate both in the membrane and in the solubilized form, we took advantage of this interaction in the purification of the enzyme. For comparison, we also undertook the purification of hyaluronidase-treated enzyme. In a typical experiment, digitonin extracts were prepared from crude membranes of hyaluronidase-treated and -untreated cells. The protein concentration chosen for the detergent extraction was high (about 20–30 mg/ml) in order to obtain as much total activity as possible in a small volume (2–3 ml) for gel filtration chromatography. However, the digitonin-to-protein ratio was maintained at optimal, 0.7. Each extract was then applied to a column of Sepharose CL-6B equilibrated with sodium phosphate buffer containing 0.1% digitonin and 10% glycerol (buffer A). The presence of 10% glycerol was found to be essential for maintaining the stability of the enzyme activity during all further steps of purification. The solubilized enzyme from hyaluronidase-treated cells was eluted from the Sepharose CL-6B in a slightly included position with about 4-fold increase in specific activity (Fig. 4a). The solubilized enzyme from untreated cells, by contrast, was eluted from the void volume of a Sepharose CL-6B column with about a 16-fold increase in specific activity (Fig. 4b). To exploit this difference further, the untreated enzyme was chromatographed on a column of Sepharose CL-4B. The enzyme was resolved into two populations using this column (Fig. 5). Even though only 40% of the activity was recovered from the void volume, it represents a greater than 100-fold purification.

For further purification, retarded fractions containing activity from Sepharose CL-6B (hyaluronidase-treated) or fractions from the void volume of Sepharose CL-4B (untreated) columns were each pooled and diluted to 10 mM sodium phosphate and sodium chloride (all the other components remained the same). They were then promptly loaded onto hydroxylapatite columns pre-equilibrated with 10 mM sodium phosphate buffer containing 0.1% digitonin and 10% glycerol (buffer B). Almost all proteins were absorbed by the gel. Following a washing step with 2–3 volumes of buffer B, the synthetase activity from each hydroxylapatite column was eluted with the same buffer containing a stepwise increase in sodium phosphate concentration (Fig. 6). The recovery of activities from the hydroxylapatite columns was about 55% of the pooled activities from Sepharose CL-6B and 65% from Sepharose CL-4B. The enzyme from hyaluronidase-treated cells was purified about 16-fold and from untreated cells about 226-fold following this additional step. Table II shows data through all the steps of purification. Although increasing the specific activity of the synthetase preparations, hydroxylapatite did not yield a high degree of purification. However, it is a very efficient ion-exchange matrix for concentrating the enzyme by the stepwise elution method. Elution with a linear gradient of sodium phosphate resulted in enzyme activity spread over a large volume (data not shown). Other ion-exchange gels such as DEAE-Sepharose do not absorb the enzyme efficiently.

The SDS-gel electrophoretic patterns, under reducing con-
proteins were removed from the purified enzyme preparations to the following proteins: myosin (200,000); β-galactosidase (116,000); phosphorylase b (92,500); bovine serum albumin (66,000); ovalbumin (45,000); carbonic anhydrase (31,000); and lysozyme (21,500).

Elution profiles clearly show that most eluted proteins were dialyzed overnight against water and further purified using a 10% discontinuous polyacrylamide gel containing 0.1% SDS. Lane 1, 20,000 × g crude membrane (50 μg); lane 2, 186,000 × g supernatant fraction of digitonin extracts (50 μg); lane 3, enzyme fraction from hyaluronidase-treated cells (50 μg); lane 4, enzyme fraction from untreated cells (25 μg). Standards used corresponded to the following proteins: myosin (200,000); β-galactosidase (116,000); phosphorylase b (92,500); bovine serum albumin (66,000); ovalbumin (45,000); carbonic anhydrase (31,000); and lysozyme (21,500).

The distribution of labeled hyaluronate was assessed by gel filtration on Sepharose CL-2B. The elution profile obtained for labeled hyaluronate produced by membrane-bound and solubilized enzyme preparations from untreated cells is shown in Fig. 8a. Both hyaluronate species have similar profiles; a peak consisting of large material which elutes in the void volume, and a second peak spread over a large volume in a more retarded position. The hyaluronate from hyaluronidase-treated was much smaller, but those produced by the membrane-bound enzyme appeared to be larger than those from solubilized enzyme (Fig. 8b). Heparin from the solubilized enzyme from hyaluronidase-treated was small enough to be included by Sepharose CL-6B and eluted as a broad peak immediately after the void volume (data not shown). All these labeled products were identified as hyaluronate by susceptibility to hyaluronidase digestion (data not shown; see also Ref. 10).

Absorption of Synthetase-Hyaluronate Complex by Proteoglycan-Sepharose 4B—The presence of a hyaluronate molecule in the synthetase-hyaluronate complex might allow the absorption of the complex by proteoglycan-Sepharose 4B. This absorption could be used to separate the complex from other compounds present in the reaction mixture. The absorption of the complex by proteoglycan-Sepharose 4B was evaluated using a Sepharose CL-4B column. The elution profile obtained for labeled hyaluronate produced by membrane-bound and solubilized enzyme preparations from untreated cells is shown in Fig. 8a. Both hyaluronate species have similar profiles; a peak consisting of large material which elutes in the void volume, and a second peak spread over a large volume in a more retarded position. The hyaluronate from hyaluronidase-treated was much smaller, but those produced by the membrane-bound enzyme appeared to be larger than those from solubilized enzyme (Fig. 8b). Heparin from the solubilized enzyme from hyaluronidase-treated was small enough to be included by Sepharose CL-6B and eluted as a broad peak immediately after the void volume (data not shown). All these labeled products were identified as hyaluronate by susceptibility to hyaluronidase digestion (data not shown; see also Ref. 10).
absorption of the complex by an affinity ligand that binds to hyaluronic acid. For this purpose an affinity matrix was prepared by coupling a 60-kDa hyaluronic acid binding domain of rat chondrosarcoma proteoglycan to CNBr-activated Sepharose 4B in borate buffer. The matrix was tested by binding [3H]glucosamine-labeled hyaluronate which could only be eluted with a strong chaotropic agent (4 M GdnHCl). Digitonin extracts of crude membranes were prepared from hyaluronidase-treated and -untreated cells in Hepes-buffered saline as described before. Each 186,000 x g supernatant fraction obtained was applied to a small column of proteoglycan-Sepharose 4B. The results (Table III) showed that both hyaluronidase-treated and -untreated enzyme were bound to the ligand as shown by the lost of about 70% of the activity in the unbound (pass-through) materials. Direct assay of the ligand itself confirmed the presence of bound enzyme, although only 30–40% of the bound activity was accounted for. The loss of activity was presumably due to the fact that immobilized enzyme was assayed in a two-phase system as compared to solubilized enzyme in a one-phase system.

### DISCUSSION

Early attempts to solubilize hyaluronate synthetase from both procaryotic and eucaryotic cells with detergents (4, 22) and organic solvents (13, 14) were unsuccessful. Our previous experiences with detergent solubilization using Nonidet P-40 and Tween 20 showed that inactivation of the synthetase occurred, and that the inactivation curves with both detergents were hyperbolic, rather than sigmoidal as would be expected if solubilization were the cause of inactivation (23). Recently, Aloni et al. (11) have solubilized the cellulose synthetase from A. xylinum with digitonin, and Triscott and van de Rijn (13) have solubilized the hyaluronate synthetase from streptococcal protoplast membranes with digitonin. Prehm and Mausolf (12) have also reported on the solubilization of hyaluronate synthetase from streptococci using sodium cholate, while Triscott and van de Rijn (13) found that octylglucopyranoside, sodium cholate, Triton X-100, and Zwitterion 314 either inhibited or failed to solubilize the synthetase. In the course of investigating the use of detergents in the solubilization of hyaluronate synthetase from mouse oligodendroglia cells we found that Chaps, deoxycholate, octylglucopyranoside, and Triton X-100 inactivated the synthetase, since little or no activity was recovered from any of the extracts (186,000 x g supernatant or pellet fractions). The inhibitory effects of these detergents were still apparent after dialysis of the detergent extracts against buffer without detergent. As in the previously mentioned bacterial systems (11, 13) digitonin was the only detergent that was successful in solubilizing the hyaluronate synthetase from glioma cells with retention of activity. It is curious that of all commonly used detergents only digitonin was able to solubilize these membrane-bound polysaccharide-synthesizing enzyme complexes from both procaryotic and eucaryotic membrane systems. Although the mechanism of digitonin solubilization of eucaryotic membrane is thought to be by removal of sterols, this class of lipids is not found in streptococcal membranes, leaving a clear explanation of digitonin’s effectiveness wanting.

Although digitonin was successful in releasing the hyaluronate synthetase complex from plasma membranes of glioma cells, only a portion of the total activity (Table I) (50%) was recovered. Since digitonin itself was not inhibitory in the enzyme assay and little activity remained in the extracted pellet, only about one-half of the enzyme was apparently solubilized in an active form. During the solubilization procedure and in subsequent purification steps, glycerol was required for maintaining the hyaluronate synthetase in an active state. It was anticipated that some agent would be required for stabilizing the enzyme activity, as we have previously shown that glycerol was essential during extraction and purification of the PAPS synthesizing enzyme system (composed of ATP sulfurylase and adenylyl sulfate kinase activities) from rat chondrosarcoma (24) and that the galactosyltransferase I involved in chondroitin sulfate biosynthesis required phospholipids for maintenance of activity following solubilization with Nonidet P-40 (25). Triscott and van de Rijn (13) showed that solubilized hyaluronate synthetase from streptococcus required phospholipids, in particular cardiolipin, for optimal expression of activity. Despite repeated attempts we were unsuccessful in reconstituting digitonin-solubilized hyaluronate synthetase from glioma cells with a range of phospholipids and lysophospholipids using the dialysis method. Indeed, the dialysis step is particularly destructive to the enzyme and resulted in loss of 50% or more activity.

Investigations into the size of the product of the solubilized hyaluronate synthesizing enzyme system showed that high molecular weight hyaluronate (107 daltons) was made (Fig. 2), indicating that the solubilized enzyme was as efficient as the membrane-bound enzyme (10). It was previously shown that treatment of oligodendroglia cells in culture with hyaluronidase prior to harvesting and assay of hyaluronate synthetase in the washed crude membrane resulted in 3–4-fold stimulation of activity (7). Furthermore, hyaluronidase treatment caused a change in the apparent steady-state kinetic patterns of double-reciprocal plots from intersecting lines for membranes from untreated cells to a family of parallel lines for treated cells. These changes were believed to be due to the conversion of primer hyaluronate to a shorter.

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**Table III**

Absorption of hyaluronate synthetase complex by proteoglycan-Sepharose 4B

| Fraction                  | Applied Total activity | Unbound | Bound | qpm/h |
|---------------------------|------------------------|---------|-------|-------|
| A. Hyaluronidase-treated  |                        |         |       |       |
| Proteoglycan-Sepharose 4B | 91,400 (100%)          | 33,200 (33%) | 20,300 (22%) |       |
| B. Untreated              |                        |         |       |       |
| Proteoglycan-Sepharose 4B | 52,300 (100%)          | 15,600 (30%) | 16,100 (31%) | None |
| Sepharose 4B              | 50,200 (100%)          | 48,700 (97%) |       |       |

1. L. H. Philipson and N. B. Schwartz, unpublished observations.
better acceptor in treated cells (7). Both of these features were also observed with the solubilized enzyme system from hyaluronidase-treated cells (Fig. 9; Table I), and demonstrate that the solubilized enzyme system shared the same regulatory properties as that of the membrane-bound synthetase. The solubilized synthetase system, however, differed kinetically from the membrane-bound system in two interesting aspects. It was more sensitive to substrate inhibition; UDP-GlcUA or UDP-GlcNAc in concentrations higher than 0.1 or 0.2 mM, respectively, were inhibitory. Furthermore, the optimal ratio of UDP-GlcNAc to UDP-GlcUA for maximal activity was about two, which was 10-fold lower than for the membrane-bound enzymes (10). Both of these features could be attributed to the fact that the solubilized enzyme system is more accessible to the substrates.

The present studies showed that the hyaluronate synthetase is bound to hyaluronic molecules both in membrane and in solubilized form. This interaction is most convincingly shown by the absorption of the synthetase-hyaluronate complex to an affinity matrix using the hyaluronate binding domain of rat chondrosarcoma proteoglycan as ligand (Table III). In untreated enzyme preparations the bound hyaluronate is very large (Fig. 8a), causing the synthetase-hyaluronate complex to be eluted in the excluded volume of Sepharose CL-6B (Fig. 4b). Still, approximately 40% of the synthetase-hyaluronate complex was large enough to be excluded from Sepharose CL-6B (Fig. 5), and this enzyme fraction was purified 229-fold following a hydroxylapatite step (Table II). The proteoglycan-Sepharose 4B matrix was not employed for purification because the bound synthetase-hyaluronate complex could only be eluted with a strong chaotropic agent such as 4 M GdnHCl which also dissociated the complex and inactivated the enzyme. For comparison, we examined the hyaluronidase-treated enzyme in a similar manner. As expected, the nascent-bound hyaluronate was smaller than that from untreated enzymes (Fig. 8b). As a consequence, the hyaluronidase-treated synthetase-hyaluronate complex was eluted in the included volume of Sepharose CL-6B (Fig. 4d), resulting in a lower fold purification, because of overlap with contaminant proteins. Surprisingly, bound hyaluronate from hyaluronidase-treated cells was larger from membrane-bound enzyme compared to solubilized enzyme (Fig. 8b). These findings suggest that digitonin preferentially solubilizes hyaluronate enzyme complex with shorter hyaluronate or that the nascent hyaluronate is shortened during the solubilization procedure. However, it is not possible to rule out that shorter hyaluronate enzyme complexes are more stable in the solubilized form than the longer species; during the labeling procedure nascent-enzyme complexes are more stable in the solubilized form. This should eventually lead to a more complete understanding of the mechanism of hyaluronate synthesis.

In the studies of Triscott and van de Rijn (13), digitonin-solubilized hyaluronate synthetase complexes from streptococcal membranes eluted from a Sepharose CL-6B column close to the void volume, in a slightly retarded position, even though the membranes were not treated with hyaluronidase prior to extraction with the detergent. This seems to indicate that hyaluronate primers in the streptococcal system were relatively small compared to untreated preparations from glial cells. This partly explains the significantly (about 1000-fold) higher specific activity of the enzyme system found in Streptococcus, since smaller primers are more efficient for polymerization (7).

As shown in the photograph presented in Fig. 7, a significant reduction in protein is observed during the purification procedure. However, the enzyme preparation was not pure enough to ascertain which protein bands might constitute the hyaluronate synthetase, since it is likely that the enzyme is made up of a number of polypeptides. Prehm and Masuolf (12) introduced an interesting method for the purification of hyaluronate synthetase based on the property of the bound nascent hyaluronate. Unfortunately, we were unable to purify the hyaluronate synthetase from glial cells using that method, since the cetylpyridinium chloride precipitates were found to contain numerous proteins when assessed by SDS-polyacrylamide gel electrophoresis (data not shown). Mian (9) purified a high M, phosphoprotein (M, 442,000) composed of three subunits with four polypeptides of varying molecular masses from Nonidet P-40-solubilized plasma membranes of cultured human skin fibroblasts that may be a constituent of the membrane-bound hyaluronate synthetase complex. However, in none of these reports has any polypeptide been shown to exhibit hyaluronate synthetase activity. One way to ascertain the molecular nature of the synthetase complex indirectly is by affinity labeling with periodate-oxidized nucleotide sugar as previously done (12). However, our experience with affinity labeling of glioma cell plasma membranes with O-[3H]UDP-GlcUA resulted in a half-dozen labeled protein bands indicating that this affinity analog is rather nonspecific. An alternative approach involves the use of suitable inhibitors, some of which are currently under synthesis in our laboratory. This approach in combination with the ability to obtain a highly active solubilized enzyme system should allow the rapid identification, purification, and characterization of the various regulatory and catalytic components of the hyaluronate synthetase complex. This should eventually lead to a more complete understanding of the mechanism of hyaluronate synthesis.

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