In order to provide some insight into the mechanism of hyaluronate synthesis, the subcellular localization of the synthetase system for hyaluronate was determined in eukaryotic cells. The mouse oligodendroglioma cell line G26-24, which produces copious amounts of hyaluronate in culture, was chosen as a system for these studies. Protease treatment and homogenization of cells followed by hyaluronate synthetase assay suggested that nucleotide-binding sites and trypsin-sensitive synthetase sites were not exposed at the outer membrane surface. Protease treatment following homogenization did result in decreased activity. Membrane fragments, prepared by gentle homogenization in iso- and hypotonic buffers, were subjected to differential centrifugation followed by several continuous sucrose equilibrium and velocity gradient systems. Hyaluronate synthetase activity co-fractionated with a plasma membrane marker in all systems, including those in which Golgi markers were separable. Treatment of intact cells in culture with several hyaluronidases resulted in a marked stimulation of cell-free synthetase activity. The stimulated activity was also found exclusively in plasma membrane-enriched fractions.

Fifty years after the isolation of hyaluronate (1) and 30 years after cell-free synthesis was demonstrated (2), the mechanism of biosynthesis of hyaluronate remains enigmatic. The importance of protein synthesis and the precise molecular events in de novo synthesis, elongation, and termination have not been elucidated (3). Even the very basic information concerning the cellular localization of hyaluronate glycosyltransferase activities is not known with certainty. In the streptococcal system the hyaluronate synthetase has been localized to the protoplasm membrane (4), but in avian or mammalian cells, reports are conflicting. Ishimoto et al. (5) found that the hyaluronate synthetase activity in avian sarcoma virus-infected chick fibroblasts was high in EDTA-suspended intact cells, but reduced following trypsin treatment. Bader et al. (7) found a lag of about 15 min between incorporation of [3H]GlcNAc and its appearance in hyaluronate in Rous sarcoma-transformed chick fibroblasts, and essentially no further lag in the appearance of labeled hyaluronate in the medium after synthesis. Appel et al. (8) found that approximately 80% of the hyaluronate synthetase activity sedimented between 600 x g and 10,000 x g. The possibility of localization in large membranes, which presumably included the plasma membrane, was noted.

Knowledge of the subcellular site of synthesis would provide a new approach with which to study the mechanisms of chain growth and extrusion into the extracellular space, and possibly explain the several differences between the properties and synthesis of hyaluronate and all the other glycosaminoglycans. In order to provide these answers and as a prelude to purifying the synthetase, we began a systematic study of the membranes possessing hyaluronate synthetase activity in eukaryotic cells. As some transformed cell lines produce large amounts of hyaluronate, one such line, the G26-24 mouse oligodendroglioma, was chosen for these studies in order to facilitate assay of the synthetase.

**EXPERIMENTAL PROCEDURES**

**Materials**—The mouse oligodendroglioma cell line (strain G26-24) was generously provided by Dr. G. Dawson (University of Chicago). The strain was originally isolated by Sundarraj et al. (9) from an immortal glial cell tumor induced by methylcholanthrene treatment of C57BL/6J inbred mice (10).

Human umbilical cord hyaluronate was a gift from Dr. Martin B. Mathews (University of Chicago). [3H]Hyaluronate, internally labeled with [14C]glucuronic acid, was prepared with isolated streptococcal membranes according to Sugahara et al. (11). Riker Laboratories supplied heparin (157 U.S.P. units/mg). Bovine γ-globulin and protein assay dye reagent concentrate were obtained from Bio-Rad Laboratories. Streptococcal hyaluronidase was a gift from Dr. A. Horwitz (University of Chicago). Bovine testicular hyaluronidase (15,000–20,000 IU/mg) was purchased from Leo, Helsingborg, Sweden. Leech hyaluronidase (crude) was a gift from Dr. B. Jacobson (Institute for Biological Sciences, Boston). Hyaluronidase from Streptomyces hyalurolyticus (2000 TRU/mg) was obtained from Miles Laboratories.

UDP-[3H]GlcUA (330 mCi/mmol), UDP-[3H]Gal (310 mCi/mmol), [3H]glucuronic acid (2 Ci/mmol) and [3H]glucosamine (sterile, 20.7 Ci/mmol) were purchased from New England Nuclear. Aqueous counting scintillant was obtained from Amersham.

All chemicals used were of the highest purity commercially available.

**Cell Culture**—Mouse oligodendroglioma (G26-24) cells were plated at a density of 1–2 × 10⁴ cells/100-mm dish in 10 ml of modified Eagle’s medium (12) supplemented with 10% fetal calf serum and 0.05 mg/ml gentamicin. Cultures were maintained at 10% CO₂ and

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95% humidity at 37 °C. Medium was replenished at day 3 and as needed until days 5-6, when cells reached a confluent density of 2 x 10^6 to 2.5 x 10^6 cells/cm^2. Cells were harvested for replating by gently rising with a wide-mouth 10-ml pipette or further processed by collection with a rubber policeman. For metabolic labeling of glycosaminoglycans, cell cultures were incubated with fresh media containing 10 μCi/ml of [3H]glucuronic acid.

Isolation of Glycosaminoglycans—Labeled glycosaminoglycans were isolated from the medium by a modification of the method of Dorfman and Ho (13). An aliquot of the medium, diluted with 3 volumes of 0.65 M NaCl, was mixed with 0.1 volume of 5% cetylpyridinium chloride and incubated for 10 min at room temperature. The pellet, washed with 0.1% cetylpyridinium chloride in 0.03 M NaCl, was dissolved in a solution of 5 M NaCl, methanol, and water (1:1:1) and precipitated by the addition of 6 volumes of ice-cold ethanol. After 10 min on ice, the pellet was collected by centrifugation, washed with ethanol, and dissolved in 0.1 M sodium acetate, pH 5. Aliquots were then incubated with and without Streptomyces hyaluronidase (5 TRU/ml, 80 °C for 18 h) followed by Sephacryl G-50 chromatography to quantitate [3H]hyaluronate.

Enzyme Treatments—Cells in monolayer culture were rinsed three times with the indicated equilibrated buffer. Cells were treated with 3 ml of 0.25% trypsin in calcium- and magnesium-free HBSS buffered with 20 mM Mops at 30 min at ambient temperature. The reaction was stopped by the addition of 1 volume of 10% fetal calf serum in media. The cells were washed again with 10% serum, then twice in buffer before harvest. Cell homogenates or isolated membranes prepared as described below were adjusted to a protein concentration of 1-2 mg/ml, and incubated at 37 °C for 1 h with a ratio of cell protein to trypsin of 10:1 (30 min at 30 °C) by a modification of the method of Carey and Hirschberg (14). For the homogenates, the reaction was stopped by the addition of a 5-fold excess of the trypsin inhibitor ovomucoid. For the isolated membranes, the reaction was terminated with a 5-fold excess of ovomucoid in 10 volumes of ice-cold buffer. The membranes were collected by centrifugation at 100,000 x g for 1 h and resuspended to the original volume.

Cells were treated with testicular, Streptomyces, or leech hyaluronidase as indicated, at the various concentrations in HBSS for 15 min at 37 °C in the presence of 10% CO2. Cells were then washed three times with ice-cold HBSS before harvest. Control cells were treated identically in the absence of enzyme.

Cell Fractionation—Suspended cells were rinsed by hand inversion in calcium- and magnesium-free HBSS, pelleted by centrifugation at 2000 x g for 10 min, cooled on ice to 4 °C, then pelleted and washed twice with 0.25 M sucrose containing 10 mM Hepes and 0.5 mM dithiothreitol, pH 7.2 (sucrose buffer). A total membrane fraction ("crude membranes") was prepared using a Branson Sonifier (Danbury, CT) with a microtip probe at 100,000 x g (SW 41 rotor). The interfaces were collected by aspiration, centrifuged at 100,000 x g for 1 h, and the resulting supernatant was centrifuged at 100,000 x g for 1 h.

In the following procedures, washed cells were disrupted by homogenization with a glass Dounce homogenizer with the tight pestle. Nuclei and large debris were removed by centrifugation at 600 x g for 10 min, cooled on ice to 4 °C, then pelleted and washed twice with 0.25 M sucrose containing 10 mM Hepes and 0.5 mM dithiothreitol, pH 7.2 (sucrose buffer). A total membrane fraction ("crude membranes") was prepared using a Branson Sonifier (Danbury, CT) with a microtip probe at 100,000 x g (SW 41 rotor). The interfaces were collected by aspiration, centrifuged at 100,000 x g for 1 h, and the resulting supernatant was centrifuged at 100,000 x g for 1 h.

The 20,000 x g pellet was further fractionated in a continuous sucrose density gradient. All sucrose solutions were prepared by weight in 10 mM Hepes and 0.5 mM dithiothreitol, and adjusted to a final osmolarity of 340 mosmol. Nuclei were adjusted to 0.34 M sucrose, and vesicles were adjusted to 0.74 M sucrose.

The 20,000 x g pellet was further fractionated in a continuous sucrose density gradient. All sucrose solutions were prepared by weight in 10 mM Hepes and 0.5 mM dithiothreitol, and adjusted to a final osmolarity of 340 mosmol. Nuclei were adjusted to 0.34 M sucrose, and vesicles were adjusted to 0.74 M sucrose.

1 The abbreviations used are: HBSS, Hank's buffered salt solution; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Mops, 2-(N-morpholino)ethanesulfonic acid; HBSS-Hepes, HBSS buffered with 25 mM Hepes; TRU, turbidity reducing units; GlcUA, glucuronic acid.

 Apparently, the production of ADP from ATP was considered a necessary condition for hyaluronate synthesis in the early literature, hence the "synthetase" nomenclature. In the interest of continuity, "synthetase" and not "synthase" is used here.
phosphatase (EC 3.1.3.9) (endoplasmic reticulum) was measured according to Aronson and Touster (18) in the presence of 1 mM EDTA. The released phosphate was determined by their modification of the procedure of Fiske and SubbaRow (20). Acid phosphatase (EC 3.1.3.2) was assayed according to Aronson and Touster (18) in the presence of 1 mM EDTA. The released phosphate was determined by their modification of the procedure of Fiske and SubbaRow (20).

Electron Microscopy—Membrane fractions were prepared as described above and fixed according to the procedure of Karnovsky (22). Sections were prepared and micrographs obtained by Dr. M. Press (University of Chicago) by the following procedure. The sections were postfixed for 1 h in 2% osmium tetroxide, dehydrated through a graded series of alcohols and propylene oxide, and embedded in epon. Thick (1 µm) and thin (70–90 nm) sections were cut with a Porter-Blum MT-2 ultramicrotome. The thin sections were stained with uranyl acetate and lead citrate (28) and examined with a Siemens Elmiskop 101 electron microscope.

**RESULTS**

**Assay of Hyaluronate Production by G26-24 Cells in Culture**—The G26-24 cell line has the unusual property of rendering the growth medium highly viscous after only 2–3 days in culture. Dawson and Kernes (23) found that hyaluronate accounted for 80% of the [3H]acetate incorporated into glycosaminoglycans by these cells. The total yield from the media was 35 mg/liter/48 h, approximately 10-fold higher than other cell lines. Slightly more than 10% of the hyaluronate as assayed in isotonic buffer at neutral pH are shown in Table I. More than 90% of the cells appeared to be the source of this difference between cells of the G26 series and other cell types.

In our studies, production of hyaluronate was confirmed and quantitated by labeling with [3H]acetate and [3H]glucosamine. Total glycosaminoglycans labeled with [3H]acetate were recovered from the media by cetylpyridinium chloride and ethanol precipitation and chromatographed on Sephadex G-50 columns before and after digestion with *Streptomyces* hyaluronidase, as shown in Fig. 1. The hyaluronidase-sensitive material accounted for 89.7 ± 6.7% (average of six determinations) of the radioactivity which eluted in the void volume. An aliquot of the medium from cells incubated with [3H]glucosamine was chromatographed on Sephacryl S-1000 columns. The single high molecular size peak trailed into the included volume. The material in this peak was unaffected by incubation with trypsin or pronase (not shown), but completely digested to low molecular weight products by leech hyaluronidase and by all other hyaluronidases employed in this study.

In order to rapidly and accurately assay hyaluronate synthetase activity in this investigation, a new assay procedure was developed. This assay is based on the incorporation of [14C]glucuronic acid into high molecular weight, hyaluronidase-sensitive material. The solubilized assay mixtures were chromatographed on identical Sephadex G-50 columns in tandem. The products of the reaction were well separated and represented less than 2% of the total substrate added. Ninety ± 5% of the radioactivity in the void volume was digestable by *Streptomyces* hyaluronidase for all enzyme fractions tested. Only 5–10% of the macromolecular radioactivity was recovered in parallel assays in the absence of UDP-GlcNAc, indicating that the assay was indeed measuring polymerization dependent on the presence of both substrates. In Fig. 2 are shown the linearity of the reaction with time and protein concentration, and the pH dependence.

**Activities with Intact Cells and Homogenates**—In order to determine whether synthetase is accessible to substrates or protease at the outer cell surface, a series of experiments was performed with whole cells and homogenates derived from them. The relative activities in whole cells and homogenates of hyaluronate synthetase, phosphodiesterase, and β-galactosidase assayed in isotonic buffer at neutral pH are shown in Table I. More than 90% of the cells were trypsin blue before sonication. Phosphodiesterase has been found useful as a primary plasma membrane marker (18) while β-galactosidase is a lysosomal hydrolase previously described in this cell line (24). Slightly more than 10% of the hyaluronate synthetase activity of the homogenate was found in intact cells, probably due to leakiness of some cells combined with uptake of substrate over the course of the incubation. Although the two marker enzymes were not assayed under their optimal conditions, the results suggest an intracellular site.
particles, in good agreement with the results of Ishimoto.

The acid phosphatase, however, showed a more even distribution; approximately 30% of the total activity did not sediment after centrifugation at 100,000 g. This confirmed the membrane-bound nature of the synthesis.

The per cent recoveries and specific activities in the three interfaces of the discontinuous sucrose gradient are shown in Table III. Although the differences are again small, the β-galactosidase showed the greatest partition into the 15/31% interface. The galactosyltransferase was differentially distributed from the phosphodiesterase activity. Hyaluronate synthetase was approximately equally distributed in the lower two interfaces, again suggesting its presence in a different membrane population than the Golgi marker.

Since hyaluronate synthetase was sedimentable at intermediate forces, a procedure was devised to exploit this feature. This method consisted of allowing cells to swell in hypotonic buffer followed by brief, gentle homogenization and rapid re-

for UDP-sugar binding (and transfer) in hyaluronate synthesis.

In another approach to probe cell surface activities, the differential sensitivity of synthetase to trypsin treatment in whole and homogenized cells was determined. For comparison, the two other enzymes examined in the previous section were included. Hyaluronate synthetase was indeed trypsin sensitive in homogenates and isolated membranes (Table II). Trypsinized whole cells, however, exhibited higher activity than their untrypsinized counterparts. This is consistent with the possibility that the activity found with intact cells is not due to surface exposed synthetase, but to the partial “leakiness” of cells to the substrates. Apparently, the cell surface was rendered more permeable to the nucleotide sugar substrates by treatment with trypsin. In contrast, the activity of the other two enzymes was not increased following trypsinization of whole cells, suggesting that they are not exposed by protease treatment.

Subcellular Fractionation—Since the preliminary experiments suggested that hyaluronate synthetase was not accessible at the external plasma membrane, attempts were initiated to subfractionate the G26-24 cells. The distribution of hyaluronate synthetase and marker activities after differential centrifugation was examined as a prelude to sucrose density fractionation. The partitions between the 20,000 X g pellet and supernatant fractions are shown in Fig. 3. Eighty per cent (±10%) of each activity was recovered from the original homogenate in these fractions. As expected, most of the phosphodiesterase and β-galactosidase activities were recovered in the pellet. The acid phosphatase, however, showed a more even distribution; approximately 30% of the total activity did not sediment after centrifugation at 100,000 X g for 1 h. The glucose-6-phosphatase and galactosyltransferase also were more evenly distributed. However, more than 80% of the hyaluronate synthetase activity was recovered in the 20,000 X g pellet and essentially all of the remaining activity was sedimentable after centrifugation at 100,000 X g for 1 h. This confirmed the membrane-bound nature of the synthetase, and suggested that it was preferentially present in large particles, in good agreement with the results of Ishimoto et al. (5) and Appel et al. (8).

Further fractionation of the pellets obtained after differential centrifugation was attempted with a variety of sucrose density gradient techniques. The distribution of activities from the 20,000 X g pellet in a continuous sucrose gradient was determined to provide an indication of the density distributions of the marker enzymes. The ω-galactosidase activities utilized routinely in the rat liver system exhibited only small differences in their density distributions in the G26-24 cells (25). By this method, an assignment for hyaluronate synthetase could not be made, as the activity distribution showed some similarities to both galactosyltransferase and phosphodiesterase.

Since the marker enzyme activities of interest had shown a narrow distribution in low density fractions, a modification of the Tabas and Kornfeld (15) method, utilized originally in the purification of rat liver Golgi membranes, was employed.

### Table I

| Enzyme* | Homogenates* / cells |
|---------|----------------------|
| Hyaluronate synthetase | 6.8 |
| Phosphodiesterase | 1.5 |
| β-Galactosidase | 8.0 |

* Assays were conducted in isotonic sucrose buffer (pH 7.2).

### Table II

| Enzyme* | Hyaluronate synthetase* | Phosphodiesterase* | β-Galactosidase* |
|---------|-------------------------|--------------------|------------------|
| Cells* | 3.1 | 1.5 | 1.0 |
| Homogenate* | 0.84 | 1.06 | 0.95 |
| Membranes* | 0.47 | 1.21 | 1.25 |

* Cells in monolayer culture were treated with 0.25% trypsin. Homogenates and membranes were treated with trypsin at a ratio of cell protein to trypsin of 10:1 (14).

* Cells were assayed in isotonic sucrose buffer (pH 7.2).

* Prepared by brief sonication and assayed as described under “Experimental Procedures.”

* Prepared by flotation method in sucrose gradients (17).

![Fig. 3. Distribution of activities between the 20,000 X g pellet and supernatant.](http://www.jbc.org/)

### Table III

| Protein or activity | Interface |
|---------------------|-----------|
| Protein | 15/31% | 31/34% | 34/36% |
| Phosphodiesterase | 7.0 | 28.1 | 64.9 |
| β-Galactosidase | 10.0 | 40.0 | 59.0 |
| Galactosyltransferase | 21.0 | 29.0 | 50.0 |
| Hyaluronate synthetase | 6.5 | 47.1 | 60.6 |

* Sucrose concentration (w/w); see “Experimental Procedures.”

* Per cent recovery for fraction.

* Specific activity relative to homogenate.
equilibration to isotonic conditions. Initial centrifugation was increased from $600 \times g$ for 10 min to $1,000 \times g$ for 10 min. This pellet and the following one ($20,000 \times g$ for 20 min) were subjected to discontinuous sucrose gradient fractionation as shown in Fig. 4. All the enzyme activities and total protein were distributed approximately equally between the low force pellet and supernatant, perhaps indicative of the gentle homogenization conditions. Phosphodiesterase and hyaluronate synthetase under these conditions yielded strikingly similar distribution patterns that were unlike that of the galactosyltransferase activity (Fig. 4A).

Electron micrographs of the various pellets and interfaces showed some differences in the ultrastructure of membrane populations (not shown). Typically, material collected from interfaces 1 and 2 (0.25 M/20% and 20/40%) contained primarily smooth vesicles and membrane fragments. In contrast, interface 3 (40/50%) contained granular vesicles and fragments indicating the presence of bound ribosomes, and also a greater number of osmophilic organelles. The membrane preparations from untreated or hyaluronidase-treated (see below) cells were indistinguishable.

**Stimulation of Synthesis Activity by Hyaluronidase Treatment**—A frequent observation made while preparing cells for homogenization was that cell pellets were loose and fluffy, in contrast to tight pellets obtained with fibroblasts or other cells that did not produce copious amounts of hyaluronate. The possibility that the loose pellet was cell-associated hyaluronate (as observed in hyaluronate-producing streptococci (26)) which might be interfering with fractionation techniques was considered. Therefore, cells in monolayer were treated with hyaluronidase before harvest. Untreated and trypsinized cells were incubated and processed in parallel since it has been shown that trypsin also removes at least some hyaluronate from the surface of eukaryotic cells in culture (27). The homogenates of hyaluronidase-treated cells were unexpectedly found to have approximately 4-fold higher specific activity of hyaluronate synthetase, but all other activities were unaffected, as shown in Table IV. The activities of all enzymes from the trypsinized cells were similar to those from controls (see also Table II).

The fractionation of the hyaluronidase-treated cells on discontinuous sucrose gradients, shown in Fig. 4, A' and B', was conducted simultaneously with control and trypsinized cells (not shown). While the distribution of protein and other enzyme-specific activities were similar to that of controls, the specific activity of hyaluronate synthetase was elevated in each interface. However, the specific activity relative to that of the homogenate was increased by 25% in interface 2 (20/40%) sucrose and decreased by 50% in the other gradient fractions. Interface 2 consistently had the highest phosphodiesterase specific activity as well, emphasizing the co-distribution of this marker enzyme and the stimulated synthetase activity.

**DISCUSSION**

Although production of hyaluronate by cells of neuronal origin in tissue culture was conclusively demonstrated over a decade ago (13), the present work is the first demonstration of the hyaluronate synthetase in a neuronal line. The G26-24 cell line has several features which are useful for cell fractionation studies. The cells are not particularly fastidious, and grow quickly to high densities. Since the cells do not produce a fibroblastic extracellular matrix, they are readily dislodged from tissue culture plates with minimal cell breakage, and therefore do not require treatment with protease or chelators which might perturb the cell surface. Finally, this line produces large amounts of hyaluronate but small amounts of...
other glycosaminoglycans.

Previous work has suggested that the hyaluronate synthetase may be located in the Golgi apparatus (6), in the manner of a typical glycosyltransferase, or that the synthetase may be located at the plasma membrane (5). While there is no precedent for an extracellular polymer to be synthesized at the latter location in mammalian systems, there does exist evidence to suggest that hyaluronate may not be synthesized in the manner suggested by the other glycosaminoglycans. Synthesis of hyaluronate is unique in that it is essentially unperturbed by inhibitors of protein synthesis (3). Although the structure of hyaluronate is similar to the other glycosaminoglycans, it is uniquely not subject to a variety of postsynthetic modifications, including uronic acid epimerization, N-deacetylation, and N- and O-sulfation, reactions that presumably occur in the Golgi and smooth membranes. Exogenously added chain initiators do not initiate hyaluronate synthesis. The use of oligosaccharides as acceptors, which was the most direct and conclusive means of elucidating the biosynthetic pathways of chondroitin sulfate and heparin, has been unsuccessful in the investigation of hyaluronate biosynthesis, despite the repeated attempts of several investigators over the last two decades (3, 29). At the least, these problems suggest that either a different synthetic mechanism or a separate compartmentation, or both, may obtain for hyaluronate.

In view of these considerations, an examination of the subcellular location of the synthetase was undertaken. The possibility that hyaluronate synthetase might in fact belong to the class of ectoenzymes with functional groups located on the outer surface of the cell membrane, as suggested by Ishimoto et al. (5) was explored. Minimal synthetase activity was observed when nucleotide sugars were incubated with intact cells, as opposed to homogenates or membrane preparations. Trypsin treatment of intact cells did not destroy activity, as did treatment of homogenates. Prehm (29) has also reported trypsin sensitivity of the synthetase in membrane preparations in a teratocarcinoma cell line. Unfortunately, we have not been able to identify a nonpenetrating reagent which preferentially inactivates the synthetase in whole cells, in contrast to the stimulation seen after hyaluronidase treatment of cells in culture. Thus, the results of homogenization and trypsin treatment of whole cells suggest that both the nucleotide sugar-binding sites and protease-sensitive sites of the synthetase are located intracellularly.

Upon homogenization of cells, essentially all of the hyaluronate synthetase activity was sedimentable by ultracentrifugation or at moderate forces (20,000 × g for 20 min), confirming its membrane-bound nature. The partition of the synthetase between pellet and supernatant fractions after centrifugation correlated with a plasma membrane marker and not with a glycosyltransferase Golgi marker, although optimal separation of the various membrane marker activities in sucrose gradients proved problematic. Even in continuous sucrose gradients where the several marker enzymes had similar equilibrium densities concentrated at 1.14 g/ml (30 to 36% sucrose), the distribution of hyaluronate synthetase was most similar to that of alkaline phosphodiesterase. The narrow distributions may have been due to the high amounts of glycosphingolipids, particularly galactosyl- and glucosylceramide, sulfatide, and ganglioside (GM1) produced by these cells, which is characteristic of their neuronal origin (24).

Better separation was achieved by nonequilibrium velocity sedimentation in discontinuous sucrose gradients. In these experiments, the most striking separations were obtained when the cells were subject to very gentle homogenization in hypotonic buffer. One thousand × g pellets were prepared to take best advantage of the previous observation that both hyaluronate synthetase and phosphodiesterase activities were found to sediment preferentially at low to intermediate forces, presumably in large membrane fragments. Intact cells, nuclei, and very large cellular debris were expected to sediment at higher densities in the gradient than liberated membranes. Analysis of these gradients showed quantitative similarity between the hyaluronate synthetase and phosphodiesterase activity in smooth membranes recovered in the 20/40% sucrose interface. Thus, by several different methods, hyaluronate synthetase was recovered in plasma membrane-enriched fractions.

Treatment of cells in monolayer culture with hyaluronidases produced an immediate and sustained increase in the activity of the synthetase, suggesting direct communication of cell surface conditions to the enzyme. This modified enzyme allowed an additional criterion with which to probe the localization of hyaluronate synthesis. To our knowledge, this effect of hyaluronidase treatment on intact cells has not previously been reported. Further analysis of the hyaluronidase effect and a novel kinetic model for hyaluronate synthesis will be presented subsequently.

In conclusion, evidence has been presented for the localization of hyaluronate synthetase at the inner surface of the plasma membrane. The presence of the synthetic apparatus for a glycosaminoglycan in the plasma membrane is novel, but does help explain several of the enigmas concerning the properties and biosynthesis of hyaluronate. If the hyaluronate molecule is not an intracellular product, then the lack of involvement of protein synthesis and any of the postsynthetic modification reactions common to the other glycosaminoglycans is immediately explained. Synthesis and extrusion directly from the plasma membrane also obviates the necessity for a packaging or secretion mechanism for these extremely large (>10^6 Da) and voluminous molecules. Furthermore, in those cells and tissues where hyaluronate is involved in aggregated structures, such as with chondroitin sulfate proteoglycan and link protein in cartilage, the obligate association interactions may begin to occur near the site of synthesis and extrusion of hyaluronate on the membrane surface.

Whether this mechanism represents an "alternative" synthetic pathway as suggested by Goldberg and Toole (30) is unclear. Their finding that the ionophore monensin inhibits extracellular accumulation of hyaluronate in rat fibrosarcoma and 3T3 cells, but not in human articular chondrocytes or rat chondrosarcoma cells (31) may be easily explained by the interruption of transport of the synthetase, not the hyaluronate, to the cell surface. Different turnover rates of the synthetase in the various cell types would unify the apparently discrepant results. The investigation of this possibility is underway.

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