Evidence is presented that monensin-sensitive membranes, presumably the Golgi apparatus, are involved in the synthesis of hyaluronate in rat fibrosarcoma cells. Monensin caused the inhibition of incorporation of metabolic precursors into hyaluronate produced by rat fibrosarcoma cells in a concentration- and time-dependent manner. Maximum inhibition (70–80%) was obtained on treatment with 10^{-7} M monensin for 24 h. Incorporation of label into secreted hyaluronate and into that associated with the cell surface was inhibited, but incorporation into intracellular hyaluronate was not inhibited. In 3T3 cells, treatment for 24 h with 10^{-7} M monensin inhibited incorporation of label only into secreted hyaluronate. The hyaluronate-rich pericellular coat, revealed by exclusion of fixed red blood cells, was depleted on treatment with monensin under the same conditions which caused inhibition of hyaluronate synthesis in the fibrosarcoma cells. Cell proliferation, as measured by DNA content/culture and [3H]thymidine incorporation, was also inhibited in a dose-dependent manner by monensin (10^{-8}-10^{-6} M). Protein synthesis was not inhibited at these doses, nor was monensin cytotoxic as judged by a 51Cr release assay. The inhibition of hyaluronate synthesis was independent of the antiproliferative effect of monensin because it was obtained during log phase growth or confluenpy and in the presence or the absence of serum.

Hyaluronate synthesis has been shown to occur in association with membranes but the subcellular sites of initiation and elongation have not been demonstrated. Two possible locations of hyaluronate elongation are suggested by previous studies, viz. the Golgi apparatus and the plasma membrane. The former is the site of glycosylation of many complex carbohydrates including certain glycosaminoglycans (1, 2), and autoradiographic experiments suggest that hyaluronate might also be synthesized in the Golgi (3). However, Turley and Roth (4) showed that extracellular hyaluronate, bound covalently to a tissue culture substratum, was elongated by attached Simian virus-transformed 3T3 cells, suggesting that at least some of the hyaluronate synthetase of these cells is associated with the external cell surface.

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Materials—RFS1 cells were obtained from Dr. A. Dorfman (University of Chicago) and 3T3 cells from Dr. J. Keller (Chicago Medical School). DMEM, BS, HS, FBS, penicillin-streptomycin, and trypsin were all from Gibco, (Grand Island, NY). Streptomyces hyaluronidase (2000 units/mg) was from Miles Laboratories (Elkland, IN). DNA (sperm whale), hyaluronate (umbilical cord, grade III), chondroitin sulfate (shark cartilage, type C), trypsin (type XIV), trypsin (type III), and ethidium bromide were from Sigma. Sephacryl S-1000 was from Pharmacia, Inc. (Piscataway, NJ) and monensin from Calbiochem (La Jolla, CA). [35S]Acetate (57.7 Ci/mmol), [3H]acetate (2.5 Ci/mmol), [3H]thymidine (MIXTURE: NET-250), [3H]thymidine (20 Ci/mol), and [35S]Na chromate (1 mCi/ml) were all from New England Nuclear. The scintillation cocktail used was Ready Solv HP from Beckman (Fullerton, CA). All other chemicals were reagent grade except for spectrograde methanol (Fisher). [14C]Hyaluronate was prepared as described previously (10).

Culture Conditions—Cells were maintained in DMEM containing 5% BS and 5% HS plus antibiotics, 100 units/ml of penicillin and 100 µg/ml of streptomycin, at 37 °C in 5% CO2, 95% air. Cells were subcultured with 0.25% trypsin, 0.02% EDTA in PBS-CMF (37 °C, 5 min) and precipitated by adding 50% trichloroacetic acid solution. After 1 h in an ice slurry, the suspension was centrifuged (10,000 g) in a Beckman Microfuge B for 3 min and the pellet washed twice with PBS-CMF. The pellets were suspended in PBS-CMF and digested with 1 mg/ml of protease, 0.01% sodium azide, 0.01% EDTA at 50 °C for 3 or 24 h. The protease solution was preincubated at 37 °C for 5 min into mini wells (2 cm²) in 1 ml of DMEM plus antibiotics with or without 10% FBS.

DNA Content—The DNA content of cultures was determined by ethidium bromide-DNA fluorescence (11, 12). Cell pellets were suspended in PBS-CMF and digested with 1 mg/ml of protease, 0.01% sodium azide, 0.01% EDTA at 50 °C for 3 or 24 h. The protease solution was precipitated by adding 10% trichloroacetic acid solution. After 1 h in an ice slurry, the suspension was centrifuged (10,000 g) in a Beckman Microfuge B for 3 min and the pellet washed twice with 10% trichloroacetic acid. The pellet was dissolved in 100 µl of 0.2 N NaOH then acidified with 20 µl of 1.0 N acetic acid. The radioactivity in the microfuge tubes was measured by adding 1.5 ml of scintillation cocktail into the microfuge tubes and counting the tubes directly in a Beckman LS-8100.

Protein Synthesis—Cultures were incubated for 3–24 h with [14C]Aminoacid mixture (1 µCi/ml), then the medium and cells were processed and counted as described previously (10).

Materials and Methods

The abbreviations used are: RFS, rat fibrosarcoma; DMEM, Dulbecco’s modified Eagle’s medium; BS, bovine serum; FBS, fetal bovine serum; HS, horse serum; PBS-CMF, phosphate-buffered saline-calcium magnesium free.
harvested. Aliquots from each were mixed with 0.1% bovine serum albumin in PBS and then with trichloroacetic acid solution (10% final concentration). The precipitate was collected, washed and radioactivity measured as described for DNA synthesis.

Cytotoxicity Assay—The $^{51}$Cr release assay was a modification of the method of MacDonald et al. (13). Briefly, the cells were incubated with 0.05 μCi/ml of $^{51}$Cr, as sodium chromate, in serum-free culture medium for 1 h. Unincorporated $^{51}$Cr was removed by washing the cells three times with serum-free culture medium. The cells were incubated with various concentrations of monensin for 24 h. The amount of $^{51}$Cr in the culture medium was measured by scintillation spectroscopy, and expressed as percentage of $^{51}$Cr released by treatment of the cells with trichloroacetic acid.

Hyaluronate Synthesis—The incorporation of labeled acetate ($^3$H, 10 μCi/ml or $^{14}$C, 1 μCi/ml) into hyaluronate was measured in three compartments: medium, cell surface (trypsinate), and intracellular (trypsin-resistant). After incorporation of isotopic precursors, the medium was removed, then the cells were washed twice and treated with 0.25% trypsin, 0.02% EDTA in PBS-CMF at 37 °C for 5 min. The trypsinate (or cell surface-derived material) was then separated.

Fig. 1. Phase contrast micrographs of RFS cells in the absence (A,C,E) and the presence (B,D,F) of monensin. A, control; B, monensin (10^-5 M, 24 h); C-F, pericellular coats visualized by the exclusion of fixed red blood cells; C, control; D, monensin (10^-5 M, 24 h); E, Streptomyces hyaluronidase digested (0.1 units/ml, 10 min); F, monensin (10^-5 M, 24 h). At lower concentrations of monensin, the coats were indistinguishable from control.
Fig. 2. Dose-dependent inhibition of hyaluronate synthesis by monensin. A, concentration dependence. After preincubating with drug for 24 h, fresh medium containing [3H]acetate (10 μCi/ml) and the same concentration of drug was added and incubation continued for another 24 h. The cpm of hyaluronate are corrected for DNA content of the cultures. Values are means ± S.E. for triplicate cultures. B, time course. Medium containing [3H]acetate (10 μCi/ml) without (●) or with (○) 10^{-7} M monensin was added to RFS cells in log phase growth at time zero. Values are the means of triplicate cultures ± S.E.

from the cells by centrifugation. All three compartments were digested with 1 mg/ml of protease for 3–24 h at 50 °C followed by boiling for 15 min to inactivate the protease. Because of the large amount of free isotope in the medium, the macromolecules in this fraction were precipitated after protease digestion with 3 volumes of 1.3% potassium acetate, 95% ethanol (~20 °C, 16–24 h). From each preparation, 200-μl aliquots were incubated at 50 °C for 3 or 24 h, with or without 1–2 units of Streptomyces hyaluronidase. The labeled glycosaminoglycans were precipitated with 40 μl of cetylpyridinium chloride solution after addition of 400 μl of carrier (0.25 mg/ml of hyaluronate, 0.5 mg/ml of chondroitin sulfate in distilled water). After a 0.5–1-h incubation at 37 °C, the samples were microcentrifuged (10,000 g, 5 min). The pellets were washed twice in 0.05 M NaCl, 0.05% cetylpyridinium chloride, then dissolved in 150 μl of methanol. The radioactivity in the microfuge tubes was measured as described for DNA synthesis.

Gel Filtration—Trypsinates and media from control and monensin-treated cultures, before and after digestion with Streptomyces hyaluronidase, were applied to a column (30 × 1 cm) of Sephacryl S-1000 and eluted in 0.5 M NaCl, 0.02 M Tris, 0.002% Na azide, pH 7.4, at a flow rate of 30–40 ml/h. Fractions (0.5 ml) were collected directly in counting vials for measurement of radioactivity. The Vₐ and Vₑ were determined with standard preparations of [14C]hyaluronate and [3H]acetate respectively.

Visualization of Hyaluronate-containing Pericellular Coats—A suspension of formalized red blood cells (750 μl, 10⁷ cells/ml of 0.1% bovine serum albumin in PBS) was added to a subconfluent 35-mm dish of RFS cells and the red blood cells allowed to settle for 10–20 min as described previously (14). The cultures were observed with a phase contrast microscope and photographed. The coats were removed by treatment with 0.1 units/ml of Streptomyces hyaluronidase at 37 °C for 30 min.

RESULTS

Inhibition of Hyaluronate Synthesis by Monensin in RFS Cells—In preliminary experiments to determine whether monensin might affect hyaluronate synthesis, RFS cells were exposed to various concentrations of monensin for 24 h then examined by the red blood cell exclusion technique for visualizing cell coats. Previous work has shown that the size of the cell surface component was decreased by 60% in the latter. Values are means ± S.E. for triplicate cultures ± S.E.

similar concentrations of monensin to those affecting the presence of hyaluronate-containing coats. The amounts of labeled hyaluronate appearing in the cell surface-associated fraction (tripsinatate) and the medium were found to decrease with increasing dose of monensin. However, incorporation into the intracellular (trypsin-resistant) hyaluronate either increased or was not significantly affected (Fig. 2A). The size of the hyaluronate secreted after monensin treatment was still very large since the cell surface and medium materials chromatographed in the void of a Sephacryl S-1000 column (data not shown).

Since 10^{-7} M monensin was the minimum concentration that caused reproducible loss of the hyaluronate-containing cell coats (Fig. 1), we used this concentration to determine the time course of inhibition of the medium, cell surface, and intracellular pools of hyaluronate (Fig. 2B). After 4 h of incubation with [3H]acetate, very little labeled hyaluronate was found in the medium of control or treated cultures, but the cell surface component was decreased by 60% in the latter. The level of inhibition in the medium was not significant
until 24 h. Subsequently, the level of hyaluronate synthesis continued to increase in the controls but approached a plateau in the treated cultures. At 48 h, incorporation in the monensin-treated cultures was only 25% of controls for the cell surface fraction and 46% for the medium. Little effect of monensin on the intracellular fraction was observed.

When RFS cells were labeled with [3H]acetate in the absence of monensin and then chased in the presence of monensin for 24 and 48 h, a 50% decrease in labeled hyaluronate was secreted into the medium (Fig. 3). No concomitant increase occurred in the cell surface or intracellular compartment. Therefore, the inhibition of hyaluronate synthesis was not due to inhibition of precursor uptake.

Inhibition of Cell Proliferation by Monensin—During the course of the above experiments, we found that monensin had a significant effect on cell proliferation and thus the data are expressed per μg of DNA. The effects of monensin on growth and DNA synthesis in the RFS cell cultures are shown in Fig. 4. The doubling time for control cultures was 8 h, compared to 48 h for cultures treated with 10^-7 M monensin (Fig. 4A). The drug at concentrations of 10^-4 and 10^-3 M did not affect cell viability as judged by the 51Cr release assay (Fig. 4B) and 3H-amino acid incorporation into protein (see below).

The Effect of Cell Density on the Inhibition of Hyaluronate Synthesis by Monensin—Because of the considerable effect of monensin on cell growth, and since cell density has been shown to affect hyaluronate synthesis in other systems (15–18), we measured the effect of 10^-3 M monensin on hyaluronate production by RFS cells at 3 different plating densities. When levels of hyaluronate in the intracellular, cell surface, and medium compartments were compared as a function of cell density (as measured by DNA content/2 cm^2 plate), a large density-dependent increase in hyaluronate production was found in all compartments of the control cultures but not in the monensin-treated cultures (Fig. 5A). Again, it can be seen that inhibition of hyaluronate accumulation by monensin is high for the medium and cell surface components, but insignificant in the intracellular fraction. Comparison of control and monensin-treated cultures between 5–7 μg of DNA/cm^2 demonstrates that monensin inhibits hyaluronate synthesis at a common cell density.

In an attempt to decrease the large density effect on hyalu-
ronate synthesis in the controls, the above experiment was repeated in the absence of serum (Fig. 5B). In this case, the final densities in the presence of monensin were only slightly less than controls (compare DNA contents for closed and open symbols in Fig. 5B), since neither set of cells proliferated rapidly during the course of the experiment. In this experiment, the inhibitory effect of monensin on incorporation into hyaluronate of the medium and cell surface, but not the intracellular pool, was seen.

Effect of Monensin on Protein Synthesis—To determine whether inhibition of hyaluronate synthesis was merely a reflection of inhibition of protein synthesis, we investigated the dose-dependent effect of monensin on [3H]aminoacid incorporated into trichloroacetic acid-precipitable pronase-digestable material. For a positive control, we used cycloheximide, an established protein synthesis inhibitor. Most of the isotope incorporated was found in the cell layer of the RFS cultures where it decreased with increasing cell density (Fig. 6). However, the RFS cells exhibited a density-dependent increase in the appearance of labeled protein in the medium. Monensin (10⁻⁷ M, 24 h) did not have any significant effect on incorporation into either cell layer or medium proteins at any density tested, whereas cycloheximide (10⁻⁵ M, 24 h) caused at least a 20-fold decrease.

Fig. 7 shows the effect of various concentrations of monensin and cycloheximide on total protein synthesis. An apparent dose-dependent inhibition of protein synthesis/culture occurs in both cases (Fig. 7A). However, when corrected for cell density (Fig. 7B), it can be seen that monensin does not have an effect between 10⁻⁸ and 10⁻⁵ M, whereas cycloheximide is inhibitory above 10⁻⁷ M.

Effect of Monensin on Hyaluronate Synthesis by 3T3 Cells—

The increase in label incorporation into hyaluronate with increasing RFS cell density was an unexpected finding since previous reports had shown density-dependent decreases in hyaluronate synthesis in other cell lines, e.g. 3T3 cells (17). In order to rule out the possibility that hyaluronate production and its sensitivity to monensin were unusual in RFS cells, we examined the effect of monensin on hyaluronate synthesis in 3T3 cells. Again, monensin had an inhibitory effect on cell proliferation and hyaluronate production. At 10⁻⁷ M, monensin caused a decrease of approximately 50% in the hyaluronate of the medium but had no effect on the intracellular fraction (Table I). At 10⁻⁷ M, the cell surface hyaluronate was not affected as measured either by biochemical analysis or coat visualization. However, the 3T3 coats were depleted in the presence of 10⁻⁸ M monensin.

**Fig. 6.** Effect of cell density on protein synthesis. RFS cells were plated at four different densities. After 24 h, the vehicle (ethanol), monensin (10⁻⁷ M) or cycloheximide (10⁻⁸ M) were added to the cultures. After a 24 h preincubation with the drugs, the cultures were incubated for 24 h with fresh medium containing the isotope (1 μCi/ml of [3H]aminoacid) and the same drug concentration as during the preincubation. The proteins of the medium and EDTA-released cell layer were precipitated with trichloroacetic acid (final concentration 10%). Each point is the mean of triplicate cultures with less than a 15% S.E. Closed symbols are controls; open symbols, drug-treated. Cell layer: control (A); monensin (a); cycloheximide (V). Medium: control (O); monensin (O). No detectable amount of labeled protein was found in the medium of the cycloheximide treated cells.

**Fig. 7.** Dose-dependent effect of monensin and cycloheximide on total protein synthesis. The same procedure as described in Fig. 6 was used, except cells were plated at a high density. The cpm values for the medium and the cells were pooled to give totals. Monensin (C); cycloheximide (>). A, protein synthesis/well, not corrected for DNA content or cell density. Values are mean ± S.E. for triplicate cultures. The insert gives the DNA content of the wells. B, protein synthesis corrected for DNA content and the effect of cell density (Fig. 6). Each value is the mean of triplicate cultures.

**Table I.** Effect of monensin on hyaluronate synthesis in 3T3 cells.

<table>
<thead>
<tr>
<th></th>
<th>Hyaluronate (cpm × 10⁴)/DNA (μg)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Medium</td>
</tr>
<tr>
<td>Control</td>
<td>22.8 ± 1.3</td>
</tr>
<tr>
<td>Monensin (10⁻⁷ M)</td>
<td>12.4 ± 0.4*</td>
</tr>
</tbody>
</table>

*P < 0.01, t test.
**Monensin Inhibition of Hyaluronate Synthesis**

The major result of the experiments described herein is that monensin inhibits hyaluronate synthesis and secretion by RFS cells. Monensin is a monovalent cation ionophore specific for sodium ion transport (19). Morphological (5-7), cytochemical (6), and kinetic (8) studies indicate that it preferentially disrupts the Golgi. However, membranes other than the Golgi may be disrupted by monensin (5) and some other Golgi-dependent processes like glycosaminoglycan sulfation (25, 26) and receptor recycling (20) are also inhibited by monensin. However, a marked difference in monensin doses is necessary for these effects. Inhibition of receptor recycling is very rapid (5-15 min) but takes higher concentrations of monensin ($10^{-6}$-$10^{-5}$ M) (20) than that required for inhibition of sulfation of glycosaminoglycans ($3 \times 10^{-6}$ M) (25) and secretion of fibronectin or procollagen ($10^{-5}$ M) (22). At $10^{-4}$ M monensin, incorporation of serine into protein is decreased in chondrocytes (23), but we were unable to demonstrate a significant effect on $^3$H-aminoc acid incorporation into protein in RFS cell cultures between $10^{-5}$ and $10^{-4}$ M monensin (Fig. 7). Mitchell & Hardingham (23) have reported that hyaluronate synthesis and secretion by rat chondrosarcoma cells is not inhibited by $10^{-4}$ M monensin, yet considerable inhibition of chondroitin sulfate synthesis and proteoglycan secretion was observed under the same conditions. They proposed that the synthesis and secretion of hyaluronate and chondroitin sulfate by chondrocytes occur along different intracellular pathways. We have confirmed the results of Mitchell and Hardingham using human articular chondrocytes. Using $10^{-6}$ M monensin for 24 h, we also found no inhibition of hyaluronate production. Neither the cell surface nor secreted populations were depleted, whereas synthesis of chondroitin sulfate was almost entirely inhibited (>90%). Although these results with chondrocytes appear contradictory to those reported herein for RFS and 3T3 cells, they point to the possibility of two pathways of synthesis and secretion of hyaluronate: one similar to that for the chondroitin sulfate side chains of proteoglycans made by chondrocytes, i.e. via the Golgi apparatus (1), and one by an unknown alternative route which may or may not involve the Golgi. Similar proposals have been made recently for glycoprotein processing and secretion (27, 28). The two putative pathways of hyaluronate synthesis and secretion may vary in their extent of utilization according to the cell type, physiological conditions, and extracellular fate of hyaluronate produced.

Even though the bulk of the synthesis of hyaluronate by RFS cells is very sensitive to monensin treatment, our results indicate that approximately 20% of the secreted hyaluronate remained insensitive to increased dosages of monensin up to $10^{-4}$ M or to prolonged drug exposure (Fig. 2). Also, with 3T3 cells, incorporation into the cell surface hyaluronate was unchanged at $10^{-4}$ M monensin while that into the medium fraction was found to decrease dramatically (Table I). A concentration of $10^{-5}$ M was required to cause depletion of the hyaluronate-rich coats around 3T3 cells compared to $10^{-7}$ M for RFS cells. In examining a wide variety of cells that exhibit these pericellular coats, we have found as much as 1000 times difference in the monensin concentrations necessary to cause depletion of the coats. These diverse findings support the above mentioned proposal that two pathways for hyaluronate synthesis and secretion may be utilized to differing extents in different cell types. One only of these pathways, one which probably involves the Golgi apparatus, would be sensitive to monensin. We conclude that this is the major pathway for hyaluronate synthesis and secretion in RFS cells.

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**REFERENCES**


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3 R. L. Goldberg and B. P. Toole, unpublished observations.
Monensin inhibition of hyaluronate synthesis in rat fibrosarcoma cells.
R L Goldberg and B P Toole


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