The Effect of Cell Density on Net Rates of Glycosaminoglycan Synthesis and Secretion by Cultured Rat Fibroblasts*

(Received for publication, April 16, 1980)

Lucjan Hronowski† and Tassos P. Anastassiades§

*From the Departments of Medicine and of Biochemistry and the Rheumatic Diseases Unit, Queen's University, Kingston, Ontario, Canada

Net rates of synthesis and secretion into the medium for hyaluronic acid and chondroitin sulfate have been calculated at varying cell densities of fibroblast-cell cultures from several tissues of the neonatal rat, utilizing an improved technique for glycosaminoglycan microquantitation. The incorporation of radioactivity from [3H]glucosamine and 35SO4 into the separated glycosaminoglycans from the cell surface and the cell pellet have also been estimated at varying cell densities. For rat muscle, skin, and heart fibroblasts, the net rate of hyaluronic acid synthesis per cell per day decreased rapidly with increasing cell densities, and the data best fit a linear relationship if plotted on semilog coordinates. The relationship of cell density to net chondroitin sulfate synthesis is more complex, but in the medium fraction a linear relationship is most closely approximated by plotting the data on linear coordinates. Glucose supplementation experiments and comparative rates of uptake of glucose from the medium by the various lines indicate that glucose depletion had only minor effects on the net synthesis and secretion of medium glycosaminoglycans. The y intercepts of linear plots of net glycosaminoglycan synthesis with varying cell density can be considered to reflect rates of synthesis and secretion at infinitely low cell densities. Under defined culture conditions, this value may be useful in characterizing the intrinsic synthetic capability of fibroblastic lines independent of the modifying influence of cell density.

Several studies have shown decreases in the rates of total glycosaminoglycan production as cultured cells progressed from low to high cell densities (1-4) by measuring the changes in the total hexuronic acid content. However, because of the small amounts of material produced by cultured cells, most of the recent studies on the rates of production of the different types of glycosaminoglycans have been performed with radioactive precursors. In agreement with the data on total glycosaminoglycan production at different cell densities (1-4), radioisotope incorporation studies using various cell types (5, 6) have shown several fold decreases in the rates of incorporation of radioactivity into hyaluronic acid as cultured cells progressed from low to high cell densities. Colin et al. (7) have observed a similar effect of cell density on rates of incorporation of radioactive label into hyaluronic acid by mouse 3T3 cells and their simian virus-transformed derivatives, using serum-free medium for the label incorporation studies. An exception to this pattern has been observed for a cultured human skin fibroblast cell line transformed with simian virus 40 (6) where the rate of label incorporation into hyaluronic acid was not dependent on cell density.

The effect of cell density on net rates of label incorporation into the sulfated glycosaminoglycans was much less (5, 6). However, while some studies have shown reduced rates with increasing cell densities for a variety of cell lines (5, 6), other studies utilizing the 3T3 cells and their simian virus 40-transformed derivatives have shown increased rates at higher cell densities (7).

Precise studies on the actual amounts of the different types of glycosaminoglycan produced at different cell densities, however, are not available. In the following study, an improved microquantitation technique (8) was applied in order to study the effect of cell density on the production of glycosaminoglycans by rat fibroblast cell lines established from different neonatal rat tissues.

EXPERIMENTAL PROCEDURES

Chemicals—d-[1,6-3H]Glucosamine hydrochloride (specific activity 39.6 Ci/mmol) and Na235SO4 (specific activity 10 to 1000 mCi/mmol) were purchased from New England Nuclear. B-(1+)-Glucose was obtained from Sigma Chemical Co. Chondroitin sulfates types A and C, hyaluronic acid, Na salt were purchased from Sigma Chemical Co. Glycosaminoglycan reference standards were kindly provided by Doctors M. A. Mathews and J. A. Cifonelli, University of Chicago. Other reagents used were as previously described (8).

Cell Culture.—The cells used in this study were obtained from outgrowths of neonatal rat tissues. Rat muscle fibroblasts (RMF) were prepared from triceps muscles as previously described (9). The cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum and supplemented with 3.7 g of NaHCO3, 0.05 g of ascorbic acid, 0.40 g of proline, 0.80 g of glycine, and 10 ml of penicillin (10,000 μ/ml) and streptomycin (10,000 μ/ml) solution per liter of media. The media, supplements, and antibiotics were purchased from Grand Island Biological Co. Rat heart fibroblasts (RHF) were obtained as follows: whole hearts were removed and placed in the culture medium. They were then cut into approximately 1-mm3 pieces and washed by passing through several changes of culture medium to remove the adhering blood cells. The heart tissue pieces were then treated like the triceps muscle tissue to obtain the RHF cells. To obtain rat skin fibroblasts (RSF), the intact skin of new born rats was washed several times with 70% ethanol, and several dorsal pieces of skin were dissected into approximately 1-mm3 pieces and treated like the triceps muscle tissue to obtain RSF cells. The fibroblast cells established from the outgrowths of the above tissues were

† This work was supported by the Medical Research Council of Canada, Grant MT-3653, and the Canadian Arthritis Society. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ To whom requests for reprints should be addressed at: Director, Queen's University Rheumatic Diseases Unit, 26 Barrie St., Kingston, Ontario, Canada K7L 3J7.

1 The abbreviations used are: RHF, rat heart fibroblast; RMF, rat muscle fibroblast; RSF, rat skin fibroblast; C-T, cell-day.
subcultured three times before being used for studies of glycosaminoglycan synthesis. All studies were performed with the cells grown on polystyrene Petri plates (Lux Scientific) in Dulbecco's modified Eagle medium containing 10% fetal calf serum, and supplemented as described above.

For studies of glycosaminoglycan synthesis at different cell densities, two experimental approaches were utilized. In the first approach, which was used for most of the studies reported here, a series of replicate plates of each cell line were prepared as follows: cells from a single healthy-looking plate at confluency were suspended with 0.25% trypsin solution (Gibco) and diluted with fresh serum-containing medium. A constant volume of this cell suspension was dispensed to each replicate plate to give the following initial cell densities: RMF-24 and RMF-48 were plated at the same cell suspension at 2000 cells/cm²; RMF-48, 5,700 cells/cm²; RSF-24, 3,100 cells/cm²; and RSF-48, 4,400 cells/cm². For each cell line, the cells were washed twice with 1 ml of serum-free medium and incubated for an additional 25 h, after which time, both pairs of cultures were labeled by refeeding them from the same batch of medium. A constant volume of this cell suspension was dispensed to each replicate plate to give the following initial cell densities: RMF-1, 5,700 cells/cm²; RSF-1, 3,100 cells/cm²; RHFl, 2,000 cells/cm²; RMF2-48, 5,700 cells/cm²; RSFI-24, 3,100 cells/cm²; and RSFI-48, 4,400 cells/cm². Cells from newly plated cells was changed approximately 12 h after plating. Subsequent refedding was performed every 24 or 48 h as indicated by the two-digit number in the name of each cell line. The RMF-, RSF-, and RHF- cells were grown for a further 2 days so that when the glycosaminoglycan cell density experiment was begun 2½ days after plating, these cells had gone through at least one division and were in their logarithmic phase of growth. With the RMF-48 cell line, the glycosaminoglycan cell density experiment was begun 2½ days after plating. For cells refed every 24 h, one replicate plate was harvested for glycosaminoglycan extraction after each 24 h of incubation, while for cells refed every 48 h, one replicate plate was harvested for glycosaminoglycan extraction after each 48 h of incubation.

The second experimental approach to the study of glycosaminoglycan synthesis at different cell densities was used with the RMF-X line. In this approach, two pairs of replicate plates were prepared from the same cell suspension where one pair had an initial cell density of 2,900 cells/cm² and the other pair, 18,800 cells/cm². These were then cultured for 2 days by which time the cell densities on the low and high density plates were 11,800 cells/cm² and 48,900 cells/cm², respectively. Both pairs of plates were refed with fresh medium and incubated for an additional 25 h, after which time, both pairs of plates were harvested for glycosaminoglycan extraction. In this second experimental approach, the time between plating and the beginning of the glycosaminoglycan cell density experiment was the same for both the low and high density cultures.

Radioisotope Labeling and Preparation of Culture Conditions—All cultures used for glycosaminoglycan extraction were grown in medium containing 0.6 to 1.3 µCi/ml of Na-35SO₄ and 0.8 to 1.0 µCi/ml of [3H]-glucosamine as indicated in each experimental section. We assure that all replicate cultures in a particular experiment were grown in the same concentration of the radioactive precursors, the cultures were labeled by refeeding them from the same batch of medium containing the radioactive precursors. Also, to assure that all glycosaminoglycans and the glycosaminoglycan precursor pools were labeled at the beginning of the growth period from which the glycosaminoglycans were extracted, the cultures were grown (unless otherwise indicated) in media containing the radioactive precursors also at the beginning of the growth period from which the glycosaminoglycans were extracted.

After the incubation periods specified above, each culture was divided into three fractions: the growth medium, the trypsin-solubilizable material (cell surface and pericellular material), and the cell pellet. The medium fraction was subdivided further into a fraction that contained cold glycosaminoglycan carrier (100 µg of hyaluronic acid and 100 µg of chondroitin sulfate in 100 µl of 0.14 M NaCl) and a fraction without carrier. Glycosaminoglycan carrier was also added to the trypsin-solubilizable material and the cell pellet fractions. These fractions were prepared as follows: the adhering cells were washed twice with 1 ml of serum-free medium and the washings combined with the medium fraction. The volume was measured and 0.2 ml of 0.25% trypsin solution (Gibco) for 20 min at 37°C and adhering cells dissolved by gently pipetting the trypsin solution over them. The cell suspension was centrifuged at a 3 ml centrate tube and combined with two 1-ml washings of the plate with serum-free media. After mixing thoroughly, the volume was measured and 0.2 ml removed and the cell number was estimated using a large chamber hemocytometer (Hauser Scientific). The remaining cell suspension was centrifuged for 5 min at room temperature using a clinical centrifuge (Setting 6) and the supernatant decanted into a 30-ml centrifuge tube (Corex). The cell pellet was resuspended in 4 ml of serum-free medium and also transferred to a 30-ml Corex centrifuge tube. After the addition of carrier glycosaminoglycan, both fractions were processed for the extraction of glycosaminoglycans, as described above.

Cell Population Growth Curves—Cell numbers on each of the plates harvested for glycosaminoglycan extraction were estimated by counting the cells in a known volume of the trypsin cell suspension as described above. Cell numbers were also determined on additional replicate plates which were treated exactly like those used for glycosaminoglycan extraction in order to obtain cell numbers at the beginning of the glycosaminoglycan cell density experiment and at other intermediate time points. These cell numbers were plotted on semilog graphs (as shown for some cell lines in Fig. 1) to obtain the cell population growth curves for each line. These growth curves were used to calculate rates of synthesis of glycosaminoglycan synthesis and average cell densities during each incubation period, as described below.

Calculation of the Net Rates of Glycosaminoglycan Synthesis and of Average Cell Densities—The rates of synthesis of the glycosaminoglycan secreted into the culture medium were expressed in terms of the amounts of the glycosaminoglycans accumulated in the culture medium per 10⁶ cells per day (i.e. per 1 million cells·days). Since the cell numbers are increasing in a complex manner throughout most of the incubation periods studied, the number of cells-days during each of the incubation periods was estimated by taking a Riemann sum (ΣC·T) over each incubation period using the cell population growth curves.

Number of cell-days during an incubation period = ΣC·T,

where C is the cell number taken from the growth curve in the middle of a short time interval T of the incubation period. This sum is then divided by 10⁶ to transform the total cell-days to the number of millions of cells·days (C·T).

The amounts of the glycosaminoglycans obtained from each incubation period were divided by the above number (C·T) to obtain the rates of synthesis per 10⁶ cells·days.

The average cell densities during each incubation period were obtained by dividing the number of cell-days (C·T) by the length of the incubation period in days (T) and the surface area (A) of the Petri plate in cm².

Average cell density (cells/cm²) = ΣC·T

T × A

Extraction, Electrophoretic Separation, Quantitation, and Characterization of the Glycosaminoglycans—The glycosaminoglycans were extracted from the three culture fractions by a modification of previously described (9) methods. The extracted glycosaminoglycans were separated by cellulose acetate electrophoresis and stained with Alcian blue (8). Radioactivity in the stained glycosaminoglycan, and for tritium and between 47 and 336% for sulfur-35 in the dual label channel are dependent on the Alcian blue present in the stained glycosaminoglycan, and for the amounts of stained glycosaminoglycan counted in this study (0.3 to 5 µg) they varied between 33 and 36% for tritium and between 33 and 27% for sulfur-35. The per cent of sulfur-35 counts overlapping into the tritium channel varied between 33 and 27%, depending on the amount of stained glycosaminoglycan present on the cellulose acetate strip. The glycosaminoglycan-Alcian blue complex of the separated glycosaminoglycans was quantitated as previously described (8) and specific activities were calculated for hyaluronic acid and chondroitin sulfate in the medium fractions that were processed in the absence of glycosaminoglycan carrier. Total incorporated radioactivity into the hyaluronic acid and chondroitin sulfate bands was estimated in the medium, trypsin-labile, and cell pellet fractions which contained, hyaluronic acid carrier, as described above. Total glycosaminoglycan synthesized and secreted into the medium was calculated from the total radioactivities incorporated into the glycosaminoglycans and the specific activities of the medium glycosaminoglycans.
Two methods were used to characterize partially the extracted glycosaminoglycans: 1) the enzymic methods using bovine testis hyaluronidase (EC 3.2.1.35) and chondroitinase ABC (EC 4.2.2.4) by a modification of previously described procedures (11); 2) in the other, an elution method, the separated glycosaminoglycans are stained in the presence of different MgCl₂ concentrations such that glycosaminoglycans with critical electrolyte concentrations lower than the salt concentration are eluted selectively off the cellulose acetate strip leaving stained on the cellulose acetate strip only the glycosaminoglycans with critical electrolyte concentrations higher than the salt concentration in the stain solution.

Glucose Analysis—Glucose in the used culture medium was measured by the colorimetric glucose oxidase procedure using reagents from Sigma glucose analysis kit number 510-A.

RESULTS

Cell Population Growth Curves—Growth properties of the different cell lines studied are illustrated in Fig. 1. Cell population doubling times during the logarithmic growth phase for cells refed every 24 h are 19 h for RHF₁-24, 22 h for RMF₁-24, and 41 h for RSF₁-24. For the RMF₁-48 line which is identical with RHF₁-24 except that it is refed every 48 h, the cell population doubling time is 27 h. The RMF₁-48 line grew slower than the other RMF lines with a minimum population doubling time of approximately 30 h.

Rates of Glycosaminoglycan Synthesis at Different Cell Densities—More than 70% of the total tritium incorporated into glycosaminoglycans in each of the cultures incubated for 24 h was found in the medium fraction with the remainder being associated with the trypsin-solubilizable and cell pellet.

![Fig. 1. Cell population growth curves for rat fibroblasts. All cells were incubated in 4 ml of Dulbecco's modified Eagle's medium containing 10% fetal calf serum. The refeeding interval is indicated by the numbers in the name of each cell line. See "Experimental Procedures" for additional details. +, RHF₁-24; ×, RMF₁-24; ○, RSF₁-24; Δ, RMF₁-48.](image)

![Fig. 2. Rates of hyaluronic acid synthesis at different cell densities by three different rat fibroblast cell lines. The rates of synthesis were calculated from the amount of material accumulated in the incubation medium during 24-h incubation periods. The amount of hyaluronic acid are expressed in terms of its hexosamine (glucosamine) content. All cultures were grown in 20.4 cm² Petri plates and were refed every 24 h from the same batch of medium. See legend to Fig. 1 and "Experimental Procedures" for additional details. C · T = cell-days; +, RHF₁-24; ×, RMF₁-24; ○, RSF₁-24.](image)

![Fig. 3. Rates of hyaluronic acid synthesis at different cell densities by two rat muscle fibroblast (RMF) cell lines. RMF₁-48 cell line is identical with the RMF₁-24 cell line in Fig. 2, whereas RMF₂-48 was derived from a different litter of rats. Both the RMF₁-48 and RMF₂-48 cells were refed every 48 h with Dulbecco's modified Eagle's medium containing 10% fetal calf serum. The rates of synthesis have been calculated from the amounts of material accumulated in the incubation medium during each 48-h incubation period. The amounts of hyaluronic acid are expressed in terms of its hexosamine content. The cell densities are the average cell densities during the 48-h incubation periods. See legend to Fig. 1 and "Experimental Procedures" for additional details. C · T = cell-days; Δ, RMF₂-48; ○, RMF₁-48.](image)

| Cell line | Slope × 10⁶ | y intercept (nmol hexosamine/10⁶ C.T cells/cm²) |
|-----------|------------|---------------------------------------------|
| RHF₁-24   | -1.24 ± 0.15 | 70.8 ± 5                                   |
| RSF₁-24   | -2.06 ± 0.21 | 645.5 ± 10                                 |
| RMF₁-24   | -2.23 ± 0.14 | 121 ± 10                                   |
| RMF₁-48   | -2.42 ± 0.13 | 106 ± 8                                    |
| RMF₂-48   | -2.04 ± 0.30 | 33.4 ± 8                                   |

The slopes and y intercepts are for the linear regression analysis calculated lines shown in these figures. Confidence intervals are ± SE.
fractions. In cultures incubated for 48 h, the tritium in medium glycosaminoglycans accounted for over 80% of the total glycosaminoglycan tritium extracted from each culture. As the cells progressed from low to high cell densities increasing proportion of the total glycosaminoglycan tritium was found in the medium fraction. The glycosaminoglycan present in the medium fraction was used in order to estimate rates of synthesis of the secreted glycosaminoglycans at different cell densities. These rates are based on material co-migrating with reference hyaluronic acid and chondroitin sulfate during separation by cellulose acetate electrophoresis. These two bands account for more than 92% of the total glycosaminoglycan by chondroitinase generated from the reference hyaluronic acid and chondroitin sulfate was degraded by bovine testis hyaluronidase, whereas the tritium extracted from the medium fraction. More than 96% of the tritium-labeled material co-migrating with reference hyaluronic acid was degraded by bovine testis hyaluronidase, and similarly, more than 96% of the tritium-labeled material co-migrating with reference chondroitin sulfate was degraded by chondroitinase ABC.

**TABLE II**

Comparison of the fit of the data shown in Figs. 2 to 5 to linear regression analysis calculated lines for linear and semilog plots.

The deviations from the calculated lines in both types of plots were expressed in terms of nanomoles of hexosamine to obtain the standard deviations of the data points from the calculated lines for both the linear and semilog plots in terms of nanomoles of hexosamine.

| Cell line | GAGa Standard deviation | Linear plot | Semilog plot |
|-----------|-------------------------|-------------|--------------|
|           | nmol hexosamine/10^6 C-T |             |              |
| RHF1-24   | Hya                     | 4.07        | 3.63         |
| RSF1-24   | Hya                     | 8.81        | 4.86         |
| RMF1-24   | Hya                     | 11.0        | 8.39         |
| RMF1-48   | Hya                     | 7.03        | 2.53         |
| RMF2-48   | CS                      | 3.44        | 2.24         |
| RMF3-24   | CS                      | 1.14        | 1.17         |
| RMF4-24   | CS                      | 0.944       | 1.24         |
| RMF5-24   | CS                      | 0.863       | 1.29         |
| RMF6-48   | CS                      | 0.862       | 1.10         |
| RMF7-48   | CS                      | 1.06        | 1.93         |

a GAG, glycosaminoglycan; Hya, hyaluronate; CS, chondroitin sulfate.

As shown in Figs. 2 and 3, the rates of hyaluronic acid synthesis decline for all cell lines as the cells progress from low to high cell densities. The slopes and y intercepts of the linear regression lines shown in Figs. 2 and 3 are summarized in Table I. The slopes which reflect the change in the rates of synthesis as cell density increases are quite similar for the RSF and RMF cell lines. In contrast, the slope for the RHF cell line is approximately one-half of that for the other lines reflecting the smaller change in the rates of hyaluronic acid synthesis as cell density increases. The y intercepts vary widely between the different cell lines reflecting the different rates of synthesis by the different cell lines at infinitely low cell densities. The largest y intercepts are observed for cell lines RMF1-24 and RMF1-48, whereas as seen in Figs. 2 and 3, the length of the refeeding interval has a small effect on the rates of synthesis, although they are slightly lower for the RHF cell line refed every 48 h throughout the entire growth period studied. The y intercept for the RMF1-48 cell line is approximately one-third of that for the RMF1-48 line, reflecting the very much lower rates of synthesis by this line. The difference between the y intercepts of the RHF1-24 and RHF2-48 is not statistically significant and these intercepts are approximately one-half as large as that for the RMF1-24 cells. However, because rates for the RHF1-24 cell line decrease less rapidly...
as cell density increases, its rate of hyaluronic acid synthesis is higher at high cell densities than the rates of the other cell lines studied.

As was seen in Figs. 2 and 3 the hyaluronic acid rates of synthesis versus cell density data approximate well a straight line on the semilog coordinates. In Table I1 the smaller synthesis versus cell density data approximate well a straight line on the semilog coordinates than on linear coordinates. However, similar statistical analysis of the chondroitin sulfate data gives smaller standard deviations of the residuals for the linear plots shown in Figs. 4 and 5. As these figures show, the net rates of chondroitin sulfate synthesis decrease with increasing cell densities, although the trend is not regular as was seen for hyaluronic acid. Table III summarizes the slopes and y intercepts of the linear regression lines shown in Figs. 4 and 5. The largest slopes or changes in the rates of chondroitin sulfate synthesis were observed for the RHF cell line, and at low densities the rates for the RHF cell lines were higher than those for the RSF line. However, at high cell densities, the rates of chondroitin sulfate synthesis by the RHF lines dropped below those of the RSF line. Of particular interest are the rates of chondroitin sulfate synthesis by the RMF1-48 and RMF2-48 cells which are quite similar and only slightly smaller than those for the RMF2-24 line. These similar rates of chondroitin sulfate synthesis for the RMF1-48 and RMF2-48 lines shown as the RMF2-48 cell line compared to the RMF1-48 cell line are different litter of neonatal rats is shown in Table IV. The rates of chondroitin sulfate synthesis by these lines as seen in Fig. 3.

**TABLE IV**

Rates of glycosaminoglycan synthesis at different cell densities by the RMF1-24 cell line

Rates of synthesis have been calculated from the amounts of material accumulated in the incubation medium during a 24-h incubation period, and the cell densities are average cell densities during this incubation period. The amounts of the glycosaminoglycans are expressed in terms of their hexosamine content. The cells were grown on 20.4 cm² Petri plates in 4 ml of Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum. For additional details see “Materials and Methods.” Two separate experiments were performed for each cell density.

| Cell Density (× 10⁶) | Rates of synthesis | Hya | Chondroitin sulfate |
|----------------------|--------------------|-----|--------------------|
| 16.4                | 7                   | 49.7, 50.7 | 14.7, 14.8 |
| 66.7                |                    | 24.5, 27.1 | 10.1, 11.7 |

"C.T = cell-days.

**TABLE V**

Rates of glucose uptake by rat fibroblasts

Rates of glucose uptake are calculated from the amount of glucose depleted from the culture medium during a 24- or 48-h incubation period (which is indicated by the numbers in the name of each cell line). Glucose in the used medium was measured by the glucose-oxidase method. See legends to Figs. 2 and 3 and "Experimental Procedures" for additional details.

| Cell line | Log growth | Glucose uptake* |
|-----------|------------|-----------------|
|           | 10⁹ C.T   | pmol/mg dry wt | pmol/10⁹ C.T |
| RHF1-24   | 5.5 (3)   | 2.0 (2) |
| RSF1-24   | 5.8 (3)   | 1.7 (2) |
| RMF1-24   | 6.3 (2)   | 4.1 (2) |
| RMF2-48   | 7.7 (3)   | 1.3 (2) |

"C.T = cell-days. Each value is an average of the values determined for two to three plates as indicated by the numbers in parentheses.

**TABLE VI**

Specific activities of glycosaminoglycans extracted from the incubation medium.

RMF1-24, RMF2-48, RSF1-24, and RHF1-24 were refed from the same batch of culture medium containing 0.3 µCi/ml of Na¹⁰⁵SO₄ and 0.8 µCi/ml of D-[1,6-³H]glucosamine. RMF2-48 was incubated with 1.3 µCi/ml of Na¹⁰⁵SO₂ and 1.0 µCi/ml of D-[1,5-³H]glucosamine. For additional details see “Experimental Procedures.”

| Cell line | Tritium specific activity | S-³⁵ specific activity |
|-----------|---------------------------|------------------------|
|           | Hya | Mean S.D. | CS | Mean S.D. | Mean S.D. |
| RMF1-24   | 7400 | 940 | 9100 | 430 | 1510 | 76 |
| RSF1-24   | 7400 | 980 | 9800 | 700 | 1570 | 168 |
| RMF1-24   | 5000 | 900 | 5900 | 900 | 1520 | 114 |
| RMF2-48   | 6200 | 860 | 8600 | 1400 | 1526 | 114 |

"S.D. = standard deviation.

Fig. 6. Double label experiment using D-[1,6-³H]glucosamine and Na¹⁰⁵SO₂ to study the effect of media glucose concentration on the specific activities of the medium glycosaminoglycans synthesized by RMF2-48 cells. Initial medium glucose concentration was 1 mg/ml. Four replicate Petri plates containing media were refed from the same batch of culture medium containing 10% fetal calf serum. For additional details see "Materials and Methods," Two separate experiments were performed for each cell density.

On the semilog coordinates, the smaller synthesis versus cell density data approximate well a straight line on the semilog coordinates than on linear coordinates. However, similar statistical analysis of the chondroitin sulfate data gives smaller standard deviations of the residuals for the linear plots shown in Figs. 4 and 5. As these figures show, the net rates of chondroitin sulfate synthesis decrease with increasing cell densities, although the trend is not regular as was seen for hyaluronic acid. Table III summarizes the slopes and y intercepts of the linear regression lines shown in Figs. 4 and 5. The largest slopes or changes in the rates of chondroitin sulfate synthesis were observed for the RHF cell line, and at low densities the rates for the RHF cell lines were higher than those for the RSF line. However, at high cell densities, the rates of chondroitin sulfate synthesis by the RHF lines dropped below those of the RSF line. Of particular interest are the rates of chondroitin sulfate synthesis by the RMF1-48 and RMF2-48 cells which are quite similar and only slightly smaller than those for the RMF2-24 line. These similar rates of chondroitin sulfate synthesis for the RMF1-48 and RMF2-48 lines shown in Fig. 5 are in contrast to the large differences in the rates of hyaluronic acid synthesis by these lines as seen in Fig. 3.

The much lower rates of hyaluronic acid synthesis by the RMF2-48 cell line compared to the RMF1-48 cell line are not due simply to the fact that this line was derived from a different litter of neonatal rats is shown in Table IV. The RMF2-24 cell line was obtained from the same group of cells as the RMF2-48 cell line. However, the RMF2-24 cell lines were refed more often and were grown in culture for 2 days after plating from a trypsin suspension before the rate study was performed. The rates of chondroitin sulfate synthesis are similar to those seen for the RMF2-24 cell line (Fig. 4) at similar cell densities. However, in the experiment shown in Table IV the rates of hyaluronic acid synthesis are about 2 times higher than those for the RMF2-48 cell line at similar cell densities (see Fig. 3).

**Glucose Uptake Rates and Specific Activities**—Table V shows glucose uptake rates for the cell lines studied. As shown in Table V glucose uptake by nondividing confluent cells is much lower than by cells in logarithmic phase of growth (4).
Also, the cells that were refed every 48 h had higher glucose uptake rates than cells refed every 24 h. Largest glucose uptake rates were observed for the RMF-48 cell line such that all medium glucose was used up by these cells near and at confluency during the 48-h incubation periods. The total uptake by the cell lines refed every 24 h was never more than 35% of the glucose initially present in the medium, and the RMF-48 cells did not remove more than 55% of the medium glucose during each 48-h incubation period.

Table VI shows the mean specific activities of glycosaminoglycans extracted from the medium fractions, except for line RMF-48 where, as indicated above, glucose was depleted completely from the medium. As shown in Table VI, chondroitin sulfate sulfur-35 specific activities vary very little, not only throughout the incubation periods shown in Fig. 1 as indicated by the relatively small standard deviation, but also between the different cell lines as indicated by the very similar means for the cells refed with the same concentration of the label. In contrast the tritium specific activities of hyaluronic acid and chondroitin sulfate generally show much larger variations both between different cell lines and within each cell line as the cells progress from low to high cell densities. In addition, the tritium specific activities of chondroitin sulfate from all cell lines are considerably higher than those of hyaluronic acid. Fig. 6 illustrates the effect of changes in the medium glucose concentration on the specific activities of the glycosaminoglycans. Addition of more glucose to the medium after a 24-h incubation period has relatively little effect on the

Table VII

Comparison of the fit of the hyaluronic acid data shown in Figs. 8 and 9 to linear regression analysis calculated lines for linear and semilog plots.

| Cell line | Culture fraction | Standard deviation |
|-----------|-----------------|--------------------|
|           |                 | Linear plot | Semilog plot  |
|           |                 | dpm/10^6 C T^-1 | |
| RHF-24    | B               | 9,320        | 7,180     |
| RHF-24    | B               | 11,000       | 4,650     |
| RHF-24    | B               | 10,600       | 3,870     |
| RSF-24    | B               | 9,160        | 6,250     |
| RSF-24    | C               | 780          | 814       |
| RSF-24    | C               | 715          | 1,720     |
| RSF-24    | C               | 2,200        | 2,570     |
| RMF-48    | C               | 1,080        | 1,520     |

a B = trypsin-solubilizable (cell surface and pericellular); C = cell pellet; dpm = disintegrations per min.

tritium specific activity of hyaluronic acid, although that of chondroitin sulfate is reduced significantly compared to the specific activities of the control. The additional glucose has no effect on the sulfur-35 specific activities or on the amounts of the glycosaminoglycans synthesized as shown in Fig. 6 (13). Synthesis and secretion of either glycosaminoglycan is not affected by glucose supplementation (Fig. 7). It should be noted, however, that the medium glucose concentration changes relatively little for the cell lines refed every 24 h. In addition, the medium glucose concentrations for each of these lines are similar at corresponding cell densities. Thus, an explanation for the large differences in the means of the tritium specific activities between the RMF-24 line and the RHF-24 and RSF-24 lines as due simply to differences in medium glucose concentration seems unlikely.

Hyaluronic Acid Radioactivity in the Trypsin-solubilizable and Cell Pellet Fractions—Statistical analysis of both linear and semilog plots of the hyaluronic acid tritium versus cell density data for RMF-24, RHF-48, RHF-24, and RSF-24 cell lines is summarized in Table VII. Hyaluronic acid radioactivity versus cell density data from the trypsin-solubilizable fraction give a better straight line relationship when plotted in semilogarithmic coordinates. However, hyaluronic acid radioactivity from the cell pellet gives a better fit in linear coordinates.

As shown in Fig. 8, hyaluronic acid in the trypsin-solubilizable fraction decreases sharply as cell density increases. The slopes of RSF-24, RHF-24, and RMF-48 are similar. However, the slopes for the RHF-24 line is about one-half of that for the other cell lines. This pattern therefore very closely parallels that seen in Figs. 2 and 3 for hyaluronic acid secreted into the culture medium. The higher y intercepts for the RHF-24 and RSF-24 cell line relative to that of the RMF-24 line seen in Fig. 8 parallels the much higher specific activities of hyaluronic acid secreted by the former cell lines.

The situation is somewhat different for hyaluronic acid tritium associated with the cell pellet as shown in Fig. 9. The RMF cell lines have larger amounts of hyaluronic acid tritium at low cell densities than the RHF and RSF cell lines. However, the hyaluronic acid radioactivity decreases much more sharply for the RMF lines so that at high cell densities less
hyaluronic acid tritium is found in these lines than in the RHFI-24 and RSFI-24 cell lines.

Chondroitin Sulfate Sulfur-35 Radioactivity in the Trypsin-solubilizable and Cell Pellet Fractions—Statistical analysis of linear and semilog plots of the chondroitin sulfate sulfur-35 versus cell density data for the trypsin-solubilizable and cell pellet fractions is summarized in Table VIII, and as shown, the standard deviations of the residuals follow the same pattern for chondroitin sulfate sulfur-35 as for hyaluronic acid tritium from these two fractions. The chondroitin sulfate sulfur-35 versus cell density data for the trypsin-solubilizable fraction gives a better straight line relationship when plotted on semilog coordinates, while for the cell pellet fraction smaller standard deviations of the residuals are obtained in linear coordinates, at least for the RMF cells. As shown in Fig. 10, chondroitin sulfate sulfur-35 radioactivity in the trypsin-solubilizable fraction decreases for all cell lines except for the RHFI-24 cell line where it changes little as cell density increases. The rate of decrease is similar for RMFI-24 and RSFI-24 lines. For RMFI-48 there is less chondroitin sulfate sulfur-35 associated with this fraction than for RMFI-24 at all cell densities.

In the trypsin-solubilizable fraction there was also a consistent zone of radioactivity that co-migrated with heparan sulfate on cellulose acetate electrophoresis. This zone was not characterized further and changed very little with increasing cell densities.

The changes in cell pellet chondroitin sulfate sulfur-35 as shown in Fig. 11 are more complex and a simple linear relationship with cell density is observed only for the RMFI-48 cell line. For both the RHFI-24 and RSFI-24 lines there is an initial phase of increase with increasing cell density followed by a decrease as cell density increases further. The pattern for the RMFI-24 line is different yet in that the cell pellet chondroitin sulfate sulfur-35 radioactivity changes very little throughout the growth phase followed by a sharp drop, so that at confluence these amounts are similar for both the RMFI-24 and RSFI-48 cell lines. Similar patterns have been observed with the cell pellet chondroitin sulfate tritium radioactivities (data not shown).

DISCUSSION

Using improved methodology for quantitation of microgram amounts of the glycosaminoglycans (8), the present study shows large decreases in the hyaluronic acid synthesized as cell density increases. Similar changes have been observed previously for a variety of cell lines, using radioisotope incorporation rates to estimate rates of synthesis at different cell densities (5, 6). However, with the present more precise meth-

**TABLE VIII**

| Cell line | Culture fraction* | Linear plot | Semilog plot |
|-----------|------------------|-------------|--------------|
| RHFI-24   | B                | 434         | 433          |
| RSFI-24   | B                | 310         | 240          |
| RMFI-24   | B                | 300         | 221          |
| RMFI-48   | B                | 196         | 112          |
| RMF1-24   | C                | 220         | 238          |
| RMF1-48   | C                | 77          | 92           |

*B = trypsin-solubilizable (cell surface and pericellular); C = cell pellet; dpm = disintegrations per min.
Effect of Cell Density on Glycosaminoglycan Synthesis

It was shown that the synthesis rates plotted against cell density give a better fit to a straight line relationship when plotted in semilogarithmic coordinates. In addition both the absolute rates and the effects of cell density on these rates is highly dependent both on the culturing conditions and on the tissue source from which the fibroblast cells are derived. This is illustrated with line RFM-X which was studied under varying culture conditions (Table IV, see also “Results”). In primary cultures of fibroblastic connective tissue cells derived from organs such as the muscle and skin of neonatal animals, the term “line” is used here in a qualified sense and only indicates the tissue of origin and the fact the fibroblastic cells grew out of the tissue during a given time interval. The possible genetic heterogeneity of such primary fibroblastic lines and the selection pressures by culture conditions have been discussed previously (9, 22). However, that the changes by secreted glycosaminoglycan represent actual decreases in the rates of synthesis rather than increased degradation rates as cell density increases is supported by the fact that similar large decreases were observed for hyaluronic acid in the trypsin-solubilizable (cell surface and pericellular) and cell pellet fractions. In addition, Truppe et al. (14) have observed for a variety of cells, very low or insignificant rates of uptake of hyaluronate added to the culture medium. Similar results were also observed for neonatal rat muscle fibroblasts (9).

Very similar slopes were observed for the different RMF and the RHF cell lines despite the fact that at similar cell densities the absolute rates of synthesis differ widely between these cell lines (Figs. 2, 3, and Table I). It is also interesting that within the experimental error the magnitudes of these slopes are twice as large as that for the RHF cell line. As has been pointed out under “Results,” it is unlikely that changes in glucose concentration can account for the different slopes of glycosaminoglycan synthesis with increasing cell density between different fibroblastic lines, although changes in medium glucose concentration have been shown to alter the rates of incorporation of radioactive glucosamine into glycosaminoglycans (20, 21). For example, it was shown that even under similar medium glucose concentrations the specific activities of the glycosaminoglycans labeled with radioactive glucosamine were 50% higher for the RHF-24 and RSM-24 cell lines compared to those synthesized by the RMF-24 cell line (Tables V and VI). These data emphasize the importance of obtaining specific activities with a reliable microquantitation method of the separated glycosaminoglycans for valid comparisons of glycosaminoglycan secretion between fibroblastic lines.

Tomida et al. (15) have demonstrated the presence of a factor in serum capable of stimulating hyaluronic acid synthetase activity and that it could be separated from factors stimulating DNA synthesis. It was also shown that this factor is probably a glycoprotein with a molecular weight of approximately 150,000 (15) and that the magnitude of stimulation was concentration-dependent (16). The large decreases of medium hyaluronic acid net synthesis with increasing cell concentration in individual lines that were observed in the present study may be at least partially explained by the fact that different relative amounts of this factor are available to the cells as their numbers increase. However, such an explanation is inadequate to explain the differences observed between the RMF-24, and RHF-24 cell lines which were cultured under identical conditions and refeed from the same batch of serum-containing medium. For these observations the only variables were the cells themselves. In this respect, the y intercepts which reflect rates of synthesis at infinitely low cell densities show that under these conditions the RMF-24 cell line can be stimulated to synthesize twice as much hyaluronic acid as the RHF-24 and RSF-24 cell lines.

A hypothesis which is consistent with the above observations must, therefore, involve differences in the cells themselves. One can speculate that the observed differences in rates of synthesis of hyaluronic acid between the different cell lines are due to different rates of exhaustion or of inactivation of the hyaluronic acid synthetase-stimulating factors which may involve prior interaction with specific cell membrane receptors, or to different turnover rates of the synthetase between the different cell lines. However, additional studies are required to explain the nature of the magnitude of the response by different cell lines at low cell densities and why the rates of synthesis decline for some cells more rapidly than for others with increasing cell density.

The observations on the changes in the amount of glycosaminoglycan associated with the trypsin-solubilizable (cell surface and pericellular) and cell pellet fractions are based only on the incorporated radioactivity into the glycosaminoglycans. However, for the cell lines considered, medium glucose varied relatively little. In addition the variations in the specific activities of the trypsin-solubilizable fractions reflect changes in the amounts of glycosaminoglycan radioactivity changes from the trypsin-solubilizable and cell pellet fractions. It is thus expected that the observed radioactivity changes in the trypsin-solubilizable fractions reflect changes in the amounts of glycosaminoglycan associated with these fractions. The above assumptions are supported by the fact that the patterns observed for chondroitin sulfate are similar whether one considers the tritium or the sulfur-35 radioactivity.

The glycosaminoglycan radioactivities from the trypsin-solubilizable fraction plotted against cell density gave better straight line relationships in semilogarithmic coordinates. In addition, the relative changes in these radioactivities between low and high cell densities were generally much greater than the changes in rates of synthesis that were observed for glycosaminoglycans from the medium fraction. The only exception to this pattern was the chondroitin sulfate radioactivity from the RHF-24 cell line which stayed essentially constant with increasing cell densities. For the other cell lines these large changes in trypsin-solubilizable glycosaminoglycan with increasing cell densities are probably related to cell crowding and decreasing rates of synthesis as the glycosaminoglycans move from the cell to the surface and into the medium. Cell pellet 35S radioactivity follows a complex pattern and the effects of cell density on cell pellet chondroitin sulfate are quite different for the different refeeding intervals (Fig. 11). For example, for the RMF-48 cells there was a steady and uniform decrease with increasing cell densities, but for the RHF-24 there was little change with increasing cell densities during logarithmic growth with a sharp drop at confluency so that both the RMF-24 and RSM-48 cells contained similar amounts of chondroitin sulfate sulfur-35. The changes for the RHF-24 and RSF-24 cells in cell pellet chondroitin sulfate sulfur-35 radioactivity also followed a complex pattern with increasing cell densities.

Previous studies have observed both increases and decreases in cellular sulfated glycosaminoglycans (17, 18). The present study shows that the nature of such observations are highly dependent on the exact culturing conditions including refeeding intervals and the growth interval over which these changes are observed.

The changes in the rates of chondroitin sulfate synthesis and secretion as indicated by the amounts of material present in the culture medium also show more irregular patterns than those observed for hyaluronic acid. The efficient uptake of proteoglycans from the culture medium has been demonstrated for a variety of cultured cells (14, 19), so that the rates...
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observed for medium chondroitin sulfate therefore probably reflect net synthesis.

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