Synthesis and Accumulation of Hyaluronic Acid and Proteoglycans in the Mouse Cumulus Cell-Oocyte Complex during Follicle-stimulating Hormone-induced Mucification*

Antonietta Salustri, Massaki Yanagishita, and Vincent C. Hascall

From the Bone Research Branch, National Institute of Dental Research, National Institutes of Health, Bethesda, Maryland 20892

In most mammalian ovaries, the cumulus cell-oocyte complex (COC) expands at the time of ovulation by depositing an extensive extracellular matrix between the cumulus cells. This phenomenon can be reproduced in vitro by culturing COCs with follicle-stimulating hormone (FSH) and serum. Biosynthesis of hyaluronic acid (HA) and proteoglycans by mouse COCs in vitro was studied using [3H]glucosamine and [35S]sulfate as metabolic precursors. Radiolabeled complex carbohydrates were analyzed by ion exchange chromatography, specific enzyme digestion followed by high performance liquid chromatography, and gel filtration. The specific activities of [3H]hexosamines in the labeled molecules were determined by measuring the incorporation of [3H] and [35S] into chondroitin 4-sulfate disaccharides. When COCs were stimulated with FSH, HA biosynthesis increased 20–30-fold between 3–12 h later when expansion occurs, reaching a maximum rate of ~780 pmol (as glucosamine)/COC/h compared with the unstimulated rate of ~26 pmol/COC/h. The final concentration of HA in the expanded COC was calculated to be ~250 μg/ml. The effects of dibutyryl cyclic AMP (Bt2cAMP) on COC expansion and HA synthesis were similar to those of FSH, suggesting that the effects of FSH are mediated by cAMP. However, FSH significantly decreased the specific activity of the incorporated hexosamines while Bt2cAMP did not.

In most mammals, oocytes in fully grown follicles are surrounded by compact layers of follicle cells to form the cumulus cell-oocyte complex (COC).1 When the circulating level of gonadotropins increases during the preovulatory period, the compact organization of the COC expands with the deposition of a mucoid material around and between the cumulus cells, a process referred to as expansion or mucification (1, 2). At ovulation, the expanded COC is released from the follicle as a viscous and elastic cell mass. Extracellular matrix components of the mucified COC appear to facilitate the pick-up of the COC by oviductal fimbria (3) and to induce changes in spermatozoa preceding the fertilization process (4). Presently, it is not known if mucification is induced directly in vivo by the gonadotropins or is mediated indirectly by the mural granulosa cells (5–7). However, gonadotropins can induce expansion of the COC in vitro if serum is present in the culture medium (8, 9). The expansion process appears to be mediated by cAMP. For example, cAMP analogues, adenylate cyclase activators, and phosphodiesterase inhibitors all stimulate mucification in vitro (10, 11), and FSH stimulates an increase in intracellular cAMP in the mouse COC in vitro (12, 13).

The ultrastructure of the extracellular matrix of the mucified COC contains a fibrillar network with a homogeneous, regular distribution that extends into the outer zona pellucida, the external coat of the oocyte (14). Specific hyaluronidases destroy this network (15) and dissociate the COC into individual cells (16). All the agents, including FSH, that induce mucification in vitro stimulate the incorporation of [3H]glucosamine as a precursor, into newly synthesized macromolecules (10). The stimulation is primarily into hyaluronic acid (HA) as determined by precipitation with cetylpyridinium chloride and susceptibility to hyaluronidase (10). Thus, HA is an important component of the expanded matrix of the COC. However, little is known about the characteristics of proteoglycans synthesized by COCs nor about their possible function in mucification. These macromolecules contain glycosaminoglycan chains covalently bound to core proteins, and

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† Visiting Associate in the Bone Research Branch, 1988. Present address: Department of Sanita Pubblica e Biologia Cellulare, Faculty of Medicine, 2nd University of Rome, via O. Raimondo, 00173 Rome, Italy.

1 The abbreviations used are: COC, cumulus cell-oocyte complex; FSH, follicle-stimulating hormone; Bt2cAMP, dibutyryl cyclic adenosine monophosphate; HEPES, N-hydroxyethylpiperazine-N'-ethanesulfonic acid; FCS, fetal calf serum; ΔΔt-HA, 2-acetamido-2-deoxy-3-O-[β-D-glucuronic acid]-D-glucose; ΔΔt-4-S, 2-acetamido-2-deoxy-3-O-[β-D-glucuronic acid]-D-glucose; ΔΔt-4-O-sulfido-D-galactose; ΔΔt-6-S, 2-acetamido-2-deoxy-3-O-[β-D-glucuronic acid]-D-glucose; ΔΔt-6-O-sulfido-D-galactose; ΔΔt-8-S, 2-acetamido-2-deoxy-3-O-[β-D-glucuronic acid]-D-glucose; hexN, hexosamine; PG, proteoglycan; HA, hyaluronic acid; HS, heparan sulfate; DS, dermatan sulfate; CS, chondroitin sulfate; GP, glycoproteins; HPLC, high performance liquid chromatography.
different types, such as those in cartilage which specifically bind to HA, contribute directly to the structure, organization, and physical properties of extracellular matrices (17). Further, PGs may influence fertilization processes directly. Addition of sulfated glycosaminoglycans to culture medium prevents fertilization in vitro for spermatozoa from several species (18, 19). Exogenous glycosaminoglycans also stimulate the acrosome reaction in vitro for spermatozoa from several species (18, 19).

In the study described in this report, we define the temporal changes in synthesis of HA and PGs during mucification of the mouse COC following stimulation in vitro with FSH or Bt$_{2}$cAMP. We show that FSH and Bt$_{2}$cAMP induce an increase of both HA and PG biosynthesis by COC cultured in vitro, and that increased net synthesis of HA, but not of PGs, correlates closely with the expansion process. A model for mucification is proposed in which cumulus cells require FSH and a factor(s) derived from the oocyte to increase HA synthesis and require a factor(s) in serum to accumulate this HA in the COC matrix.

**EXPERIMENTAL PROCEDURES**

**Materials—Guanidine HCl and urea were purchased from Bethesda Research Laboratories; [35S]sulfate (25.9 Ci/mmol) and D-[	extsuperscript{3}H]glucosamine (29.5 Ci/mmol) and D-[	extsuperscript{3}H]glucosamine were dissolved in DuPont/Nuclear Oncological Nuclear Data; Sephadex G-50, Q-Sepharose, prepacked Superose 6, and Sephacryl S-1000 from Pharmacia LKB Biotechnology Inc.; Partisil 5 PAC (0.4 × 25 cm) from Whatman. The scintillation mixture, Ready Safeguard, was obtained from Beckman; Triton X-100 from Pierce Chemical Co.; chondroitin ABC (Proteus vulgaris), heparitinase (Flavobacterium heparimarinum), and chondroitin sulfate disaccharides from Seikagaku Kogyo (Tokyo) through ICN Biochemicals; hyaluronidase (Streptomyces hyalurolyticus sp.); bovine serum albumin and buffered at pH 7.2 with 20 mM HEPES. COCs were released into the medium by puncturing large follicles. They were incubated with [3H]glucosamine and [35S]sulfate for 18 h, then the extract was transferred to a tube and brought to appropriate volume with 4 M guanidine HCl. Media samples were diluted with 500 µl (~5 volumes) of 4 M guanidine HCl buffer containing protease inhibitors. All extracts were stored frozen until further analyses.

**Evaluation of Mucification Process—**COCs were examined for cumulus mucification with a stereomicroscope. Expansion, elasticity, and resistance to mechanical disruption of the COCs were criteria for a positive response.

**Isolation of Proteoglycans—**Guanidine HCl extracts of COCs and media samples were eluted on Sephadex G-50 columns (8 ml of bed volume for 2 ml of sample) equilibrated with 8 M urea, 50 mM sodium acetate, 0.15 M NaCl, 0.5% Triton X-100, pH 7.0, to remove unincorporated isotopes and guanidine HCl (23). Eluted fractions with labeled macromolecules were applied onto Q-Sepharose columns (0.7 × 4 cm) equilibrated with the same urea buffer. After sample application, each column was washed with 5 ml of equilibrating buffer and then eluted with a NaCl gradient (0.15–1.2 M) in the 8 M urea buffer (total volume of 46 ml) with a flow rate of 15 ml/h. Fractions of 1 ml were collected and aliquots were measured for radioactivity and conductivity.

**Gel Filtration—**A prepacked Superose 6 column (1 × 30 cm) was eluted with 4 M guanidine HCl, 50 mM sodium acetate, 50 mM Tris, 0.5% Triton X-100, pH 7.0, at room temperature at a flow rate of 0.4 ml/min. Fractions of 0.4 ml were collected for analysis. A Sephacryl S-1000 column (0.7 × 35 cm) was equilibrated with 4 M guanidine HCl, 50 mM sodium acetate, 50 mM Tris, 0.5% Triton X-100, pH 7.0, at a flow rate of 3 ml/h. Fractions of 0.5 ml were collected for analysis.

**Enzyme Treatments—**Hyaluronidase digestion (30 turbidity reducing units/ml) was done in 0.1 M NaCl, 0.1 M acetate, pH 5.0, for 2 h at 37 °C. Chondroitinase ABC (0.1 unit/ml) or heparitinase (100 mU/ml) digestion of samples was done in 0.1 M Tris, 0.1 M acetate, pH 7.3, for 2 h at 37 °C.

**Quantitation of HA, PGs, and Glycoproteins—**After adding 200 µg of HA and 100 µg of bovine serum albumin to each sample, aliquots of COC extracts and media samples were eluted on Sephadex G-50 columns (2 ml of bed volume for 0.5 ml of sample), equilibrated in 0.1 M Tris, 0.1 M acetate, 0.5% Triton X-100, pH 7.3, to remove unincorporated isotopes and guanidine HCl. Macromolecules in the excluded fraction were then digested with chondroitinase ABC as described above. A portion of each digest was applied to a Sephadex G-50 column (4 ml of bed volume) equilibrated in 8 M urea, 0.15 M NaCl, 50 mM sodium acetate, 0.5% Triton X-100, pH 6.0, and eluent fractions were analyzed to determine proportions of radioactivity that was enzyme susceptible (HA and derauman sulfate disaccharides in included volume) and enzyme resistant (heparan sulfate and glyco-proteins in excluded volume). Another portion of each digest was analyzed for the proportions of the disaccharide digestion products by an HPLC procedure using Partisil 5 PAC (24) after adding Adi-3, Adi-4S, and Adi-OS disaccharide standards (5 µg each) as internal standards. The Adi-HA in each sample was derived from the enzyme digestion of the carrier HA.

For all experiments, radioactivities of samples were determined with a Beckman LS 5801. Differentiation of [3H] and [35S] activity was done by calculating [3S]/[3H] spillover into the [3H] channel using [3S] standards prepared for each set of samples.

**Statistical Analysis—**Differences between two groups of data were analyzed by Student's t test. p < 0.05 was considered significant.

**RESULTS**

**Cumulus Cell-Oocyte Complex Mucification in Vitro—**Fresly isolated mouse cumulus cell-oocyte complexes (COCs) contain 1,400 ± 200 cumulus cells compactly associated with the oocyte, Fig. 1a. This structure undergoes little change when the COCs are incubated in basal conditions (medium plus FCS) for 18 h, Fig. 1b, but undergoes extensive expansion when FSH is added to the basal medium, Fig. 1c.  

**Procedures to Identify and Quantify HA and PGs—**Isolated COCs were stimulated with FSH in the presence of FCS and incubated with [3H]glucosamine and [35S]sulfate for 18 h,

A. Salustri, unpublished data.

We are very grateful to Gary Best, National Institutes of Health Graphics Department, for preparing the photomicrographs.

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a

b

c

FIG. 1. Photomicrographs of COCs using differential interference contrast optics. freshly isolated COCs (diameter of COC \( \sim 130 \mu m \)) (a); (b) COCs incubated for 18 h in medium with 5% FCS but without FSH (note that the cumulus is not expanded, diameter of COC \( \sim 130 \mu m \)) (b); and COCs incubated for 18 h in medium with 1 \( \mu g/ml \) FSH and 5% FCS (c) (note that the cumulus is expanded, diameter of COC \( \sim 250 \mu m \)). The bar in panel is 50 \( \mu m \).

sufficient time for expansion to occur (9). A COC extract and medium sample were prepared and eluted on Sephadex G-50 columns with an 8 M urea solvent. Labeled macromolecules in the excluded fractions were then analyzed by ion-exchange chromatography on Q-Sepharose. For the COC extract, Fig. 2a, \( \sim 38\% \) of \(^3H\)-labeled macromolecules did not bind (peak 1), \( \sim 21\% \) eluted as a narrow peak during the gradient at 0.28 M NaCl (peak 2), and \( \sim 40\% \) eluted as a broad peak between 0.55–0.85 M NaCl (peak 3). Almost all of the incorporated \(^35S\) activity eluted in peak 3. In a number of different experiments, 60–80% of the labeled molecules in peak 2 were digested to small fragments by \( S.\) hyaluronidase (data not shown). Further, authentic HA added to a similar sample eluted with peak 2. Therefore, this peak contained primarily labeled HA. For peak 3, \( \sim 65\% \) of the \(^3H\) (~70% of the \(^35S\)) was susceptible to chondroitinase ABC and \( \sim 25\% \) of the \(^3H\) (~30% of the \(^35S\)) to heparitinase. Other experiments showed that most of the heparan sulfate eluted in the earlier fractions of peak 3 (data not shown). Peak 3, then, contained almost exclusively labeled dermatan sulfate and heparan sulfate PGs.

The medium sample gave a similar profile on Q-Sepharose, Fig. 2b. Peaks 1, 2, and 3 contained \( \sim 54\% \), \( \sim 11\% \), and \( \sim 35\% \) of the \(^3H\) label respectively; \( \sim 70\% \) of peak 2 was digested with \( S.\) hyaluronidase, and \( \sim 55\% \) and \( \sim 25\% \) of the \(^3H\)-labeled molecules in peak 3 were digested with chondroitinase ABC and heparitinase, respectively. For the 18-h labeling time, \( \sim 70\% \) of the total incorporated \(^3H\) and \( \sim 60\% \) of the \(^35S\) were recovered in the COC extract.

While recoveries of \(^35S\)-labeled macromolecules from Q-Sepharose were nearly quantitative, averaging between 90–98%, recoveries of \(^3H\)-labeled macromolecules were poorer, 85–95% for media samples and only 45–70% for COC extracts. Most of the missing \(^3H\) in the COC extracts bound at the top of the Q-Sepharose columns. The ion-exchange procedure, then, is useful for purifying PGs synthesized by the COC; but the low recoveries of \(^3H\), especially in the COC extracts, indicated that it gives poor yields of either HA (peak 2), or glycoproteins (peak 1) or both. Another procedure was therefore developed to address this problem.

Bovine serum albumin and HA were added to samples to facilitate recoveries, and the mixtures were eluted on Sephadex G-50 columns equilibrated with 0.1 M Tris, 0.1 M sodium acetate, 0.05% Triton X-100, pH 7.3. The recoveries of macromolecular \(^3H\) and \(^35S\) were quantitative. Aliquots of the excluded fractions were digested directly with chondroitinase ABC under conditions sufficient to digest all of the carrier and labeled HA as well as the labeled dermatan sulfate. Undigested macromolecules were removed by centrifugation after precipitation with 3 volumes of ethanol. Standard chondroitin sulfate disaccharides were added to the soluble digestion products, and the samples were then analyzed by HPLC on Partisil 5 PAC (24). For the COC extract, two \(^3H\) peaks were observed eluting with \( \Delta di \)-HA and \( \Delta di \)-4S, respectively (Fig. 3). The former contained 80–90% of the \(^3H\) in the analysis while the latter contained 10–20% as well as all of the \(^35S\).

The percent of \(^3H\) in heparan sulfate in the each sample was evaluated independently by digesting a separate aliquot with heparitinase followed by elution on Sephadex G-50 to quantify the digestion products. For the COC extract, \( \sim 5\% \) of the total \(^3H\) and \( \sim 30\% \) of the \(^35S\) was in heparan sulfate, the latter value in close agreement with the data described above for peak 3. The \(^3H\) and \(^35S\) distributions for both the COC extracts and medium samples are summarized in Table I along
Hyaluronic Acid Synthesis in Cumulus Cell-Oocyte Complexes

Comparison of ion-exchange chromatography and enzyme digestion plus HPLC procedures for quantitative determination of $^3$H-labeled macromolecules

| Analytical method                  | COC extract | Relative amount of $^3$H incorporation* |
|-----------------------------------|-------------|----------------------------------------|
| Peak 1                            | 19%         | 19% (1.9)                              |
| Peak 2                            | 60%         | 0 (5.5)                                |
| Peak 3                            | 21%         | 21% (1.1)                              |
| Total                             | 100%        | Total (1.1)                            |

Enzyme digestion + Partisil 5 PAC

| % of $^3$H in sample             | 51%         | 19% (1.9)                              |
| Peak 1                           | 49%         | 30% (1.0)                              |
| Peak 2                           | 10%         |                                       |
| Peak 3                           | 31%         |                                       |

* COCs cultured in labeling medium for 18 h with FCS and FSH; total $^3$H incorporation: ~1200 cpm/COC/18 h, with ~70% in the COC extract.

$^3$H resistant to chondroitinase and heparitinase digestion.

$^3$H in Adi-4S in chondroitinase digests.

$^3$H in Adi-4S in chondroitinase digests plus heparitinase digestion products.

Values in parentheses are ratios of values determined by the enzyme method to those determined by the ion-exchange method.

Fig. 3. Partisil 5 PAC elution profile of disaccharides derived from chondroitinase ABC digests. The peaks for disaccharide standards are shown at the top, and the radiolabeled products derived from the extract of COCs cultured with FSH and FCS are shown at the bottom. The large UV absorbing peak at the breakthrough is from the Triton X-100 in the sample.

Specific Activity Corrections—When $[3H]$glucosamine is used as a metabolic precursor, it is diluted in the cytoplasm by endogenous pathways for synthesis of hexosamines. Thus, the specific activity of the UDP-N-acetylgalactosamine precursors for glycosaminoglycan synthesis is much lower than that of the $[3H]$glucosamine in the medium, and it often varies depending upon the experimental conditions (25-28). For this reason, an indirect method was used to estimate the specific activity of the UDP-N-acetylgalactosamine pool (28). The method uses the specific activity of $[^35S]$sulfate in the medium, sa(S), combined with the $^{3H/35S}$-labeling ratio, lr, in the monosulfated Adi-4S to calculate the specific activity of the galactosamine, sa(H), in this disaccharide derived from the dermatan sulfate synthesized during the labeling period. The formula is (28):

$$sa(H) = sa(S) \times lr$$

(1)

Because UDP-galNac and UDP-glcNac are in a rapid equilibrium (29), the specific activity of glucosamine in the newly synthesized HA is assumed to be equivalent to sa(H). In the experiments reported in this paper the specific activity $^{35S}$sulfate in the medium is not significantly diluted by endogenous sulfate sources in the intracellular phosphoadenosinephosphosulfate pool, the immediate metabolic precursor for the sulfate ester in glycosaminoglycan synthesis (28). For rat granulosa cells, less than 2% of the sulfate esters on the proteoglycans is derived from cysteine or methionine when environmental sulfate in the medium is not limiting, i.e., above 0.1 mM (M. Yanagishita, J. Kimura, V. Hascall; unpublished data).
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TABLE II

| Treatment | HA | DS-PG | HS-PG | GP | Net synthesis |
|-----------|----|-------|-------|----|---------------|
| Basal     | 0.10 | 0.61  | 0.72  | 0.72  | 0.18 | 0.16          |
| FSH       | 0.03 | 0.05  | 0.06  | 0.06  | 0.36  | 0.56          |
| Bt2cAMP   | 0.02 | 1.50  | 1.00  | 0.25  | 0.36  | 0.79          |

| Treatment | 3H incorporation | sa(hexN) | Net synthesis |
|-----------|-------------------|----------|---------------|
| Basal     | 0.109 | 0.03 | 0.16 | 0.69 |
| FSH       | 0.055 | 0.03 | 0.10 | 0.46 |
| Bt2cAMP   | 0.193 | 0.06 | 0.10 | 0.51 |

*a* Calculated from equation 1 as described in the text.
*b* Values represent averages of three experiments. Coefficients of variation were less than 25%.

Fig. 5. Changes in the synthesis of 3H-labeled components in COC extracts (a) and medium samples (b) for COC cultures treated as described in Fig. 4 legend. Error bars represent 1 S.D. of the 35S-sulfate in the medium was determined to be ~37.5 μCi/μmol initially (30 μCi of 35S-sulfate/800 nmol sulfate/ml), and corrections for radioisotope decay were made thereafter. The values for sa(H) and the total incorporation, then, can be used to calculate the masses of HA and PGs synthesized during the labeling period.

Changes in Net Synthesis—An example of such an analysis is shown in Fig. 4 for samples derived from COC extracts after incubation in labeling medium for 18 h without (control), or with stimulation of mucification by FSH or Bt2cAMP. The calculated results are given in Table II and summarized in Fig. 5. It is clear that FSH decreased the specific activity of the UDP-N-acetylhexosamine precursors relative to the control, whereas Bt2cAMP did not. Thus, the values for the 3H-labeled macromolecules must be corrected for these differences in specific activity. Both FSH and Bt2cAMP increased net synthesis of all of the glycosylated components, but the largest increases occurred for HA. Even though Bt2cAMP stimulated a larger incorporation of 3H into HA (7.2 × control) than FSH (6.1 × control), the correction for specific activity differences shows that FSH increased net HA synthesis (9.9 × control) more than did Bt2cAMP (6.5 × control).

The amounts of the newly synthesized glycosylated components released into the culture medium in this experiment were also determined by the same procedure. There were no significant changes between control and treated samples for any of the glycosylated components except the small increase of DS-PG by FSH treatment (Fig. 5). For this reason, in the following experiments only data for the COC extracts are presented.

The effects of different concentrations of FSH and Bt2cAMP on the specific activity of the UDP-N-acetylhexosamines and the net synthesis of HA and PGs in COC extracts for 18-h incubations are shown in Fig. 6. Maximal levels of stimulation of synthesis were obtained with 5 ng/ml FSH and 0.5 mM Bt2cAMP, with ED50 values of ~1 ng/ml and ~0.2 mM, respectively. As noted above, HA synthesis increased much more than for PG synthesis. Both types of PGs, heparan sulfate and dermatan sulfate, showed about the same degree of stimulation. The specific activities of the Δdi-4S disaccharides for the different samples as defined by equation 1. Error bars represent 1 S.D.

Fig. 6. Changes in synthesis of HA and PG in COC extracts of cultures incubated in labeling medium for 18 h with FCS and different doses of FSH (a) or Bt2cAMP (b). The insets show the specific activity of the hexosamines (ΔH) calculated from the 3H/35S ratio of the Δdi-4S disaccharides for the different samples as defined by equation 1. Error bars represent 1 S.D.

Time Course of HA and PG Synthesis—HA and PG syn-
FIG. 7. Changes in synthesis of HA and PG during different time intervals following stimulation of COC cultures with FSH (a) or Bt2cAMP (b). Values were calculated based on averages of two observations for each time period and expressed as the ratio to control COC cultures incubated with FCS alone. For this experiment basal control levels were constant at 26, 5, and 7 pmol/h (as hexosamine) for HA, HS-PG, and DS-PG, respectively.

Fig. 8. Superose 6 elution profiles for the PG fractions (peak 3, Fig. 2) of COC cultures incubated in labeling medium for 18 h with FCS and FSH. Panels a and b show samples derived from the COC extract and medium compartments, respectively. Panel c shows a sample from the medium compartment of a COC culture incubated additionally with β-xyloside as described in the text.

Fig. 9. Sephacryl S-1000 elution profiles for radiolabeled macromolecules from COC cultures incubated in labeling medium for 18 h with FSH, and either with (a and b) or without (c and d) FCS. COC extracts (a and c) and medium samples (b and d) are shown. The dashed lines in panels a and d are the elution profiles of separate samples after digestion with Streptomyces hyaluronidase.

6–12 h. The rate then returned toward control levels during 12–18 h. The maximal increases in both dermatan sulfate and heparan sulfate PG synthesis (three to four times control) were statistically significant but were much less than for HA. Additionally, the time course of stimulation was different, with elevated synthesis of PGs sustained through the 12–18-h time period.

Characterization of PGs Synthesized by the COC—PG fractions (peak 3) were isolated from COC extracts and media samples of control and FSH stimulated COCs by chromatography on Q-Sepharose (see above). After concentration, aliquots were eluted on Superose 6 in 4 M guanidine HCl with 0.5% Triton X-100. The elution profiles for the COC extract and medium PGs of the FSH-stimulated COCs are shown in Fig. 8. The medium sample showed two major PG peaks, one excluded (Mr greater than 300 kDa) and the second centered at a $K_d$ of 0.21 (Mr, ~150 kDa). Since dermatan sulfate is predominant in the medium, these two PG peaks probably correspond to DS-PG-I and DS-PG-II previously described as synthetic products of rat granulosa cells (30, 31). The COC extract contained PG peaks with similar elution positions

### Table III

| Treatment          | COC extract | Medium | HA (pmol hexN/COC) | PG (pmol hexN/COC) |
|--------------------|-------------|--------|-------------------|-------------------|
| FCS                | 0.33 ± 0.12 | 0.70 ± 0.32 | 0.63 ± 0.50 | 4.33 ± 1.50 |
| FCS + Bt2cAMP (0.5) | 1.01 ± 0.20 | 1.40 ± 0.50 | 1.27 ± 0.23 | 10.57 ± 3.00 |
| BSA + FSH (1.9)    | 6.39 ± 2.10 | 1.01 ± 0.21 | 2.39 ± 0.70 | 10.57 ± 3.00 |

COCs were cultured in labeling medium for 18 h under the treatments indicated. Values in brackets are percentages in COC extracts and respective medium samples. Values are means ± S.D.
followed by a broad peak centered between $K_a$ 0.2-0.6. This latter peak probably contains intracellular glycosaminoglycan degradation products similar to those described for the rat granulosa cells (32, 33). The elution profiles for samples from the basal, unstimulated COCs were essentially identical. More complete analyses of the PGs synthesized by the COCs are underway. These preliminary results, however, indicate that there are no obvious qualitative changes in the PGs produced by COCs stimulated to mucify compared with unstimulated COCs.

**Effects of Nitrophenyl-$\beta$-d-xylidoside on HA and PG Synthesis on COC Expansion—$\beta$-Xylosides act as exogenous initiators for glycosaminoglycan synthesis, thereby competing with the endogenous core protein acceptors and decreasing mature PG synthesis, particularly for chondroitin sulfate/dermatan sulfate PGs (34, 35). FSH-stimulated COCs were continuously exposed to 0.5 mm nitrophenyl-$\beta$-d-xylidoside during 18 h of culture to determine if inhibition of PG synthesis affects expansion. The xyloside treatment increased total $^{35}S$ incorporation 9-10-fold. Almost all of the $^{35}S$-labeled molecules were recovered in the medium and eluted as free glycosaminoglycan chains on Superose 6, Fig. 8c (compare with 8b). The xyloside also inhibited the $^3H$ incorporation into PGs accumulated in the COC matrix to $\sim$20% of FSH-treated COC, Table III. In agreement with previous observations (36), HA synthesis was slightly inhibited (75% of FSH-treated COC) by the xyloside treatment, Table III. The xyloside treatment did not alter the FSH-stimulated COC mucification in any obvious way as assessed by light microscopy (data not shown). Thus, a significant reduction in the deposition of newly synthesized PGs in the COC matrix by the xyloside treatment did not affect the expansion of the cumulus, suggesting that PGs have little or no role in the mucification process.

**Effect of the FCS on HA and PG Synthesis and Distribution**—In the presence of FSH, but the absence of serum, cumulus expansion does not occur in vitro and the proportion of $^3H$-labeled macromolecules ($[^3H]$glucosamine as a precursor) in the medium increases (37). For this reason, the effects of FCS on net synthesis of HA and PG and on their distribution were studied in FSH-stimulated COCs (Table III). In the absence of FCS (BSA + FSH), net synthesis of PGs was unchanged while net synthesis of HA increased $\sim$60% compared to cultures with FCS (FSH + FCS). Further, the distribution of the PGs between the COC extract and the medium was unchanged. However, there was a dramatic redistribution of HA from the COC matrix into the medium, Table III and Fig. 9. After 18 h in the presence of FCS, $\sim$80% of the total labeled HA accumulated in the COC matrix. Conversely, in the absence of FCS, only $\sim$20% of the total labeled HA accumulated in the COC matrix with the remainder released into the medium, Table III. Morphological observations confirmed that cumulus expansion did not occur for COCs incubated without FCS (data not shown).

Labeled macromolecules in the cell and medium fractions for both treatments were analyzed by Sephadryl S-1000 chromatography in 4 m guanidine HCl, 0.5% Triton X-100, Fig. 9. $^3H$-Labeled HA synthesized by the COCs in the presence of FCS and extracted from the COC matrix eluted in the excluded column volume, as indicated by the sensitivity of this peak to Streptomyces hyaluronidase digestion, Fig. 9a. Similarly, the labeled HA in the medium for COCs incubated without FCS was also excluded from the column, Fig. 9d. Thus, in both conditions, the hydrodynamic size of the labeled HA molecules indicates that they have $M_r$ values greater than 2 million (38), and it is unlikely that changes in their physical properties per se account for the observed redistribution.

**DISCUSSION**

The experiments described in this report use $[^3H]$glucosamine and $[^35S]$sulfate to study the synthesis and distribution of hyaluronic acid and proteoglycans in mouse cumulus oocyte complexes stimulated to mucify in vitro. While extraction of labeled macromolecules from the COC matrix with 4 m guanidine HCl and detergent was efficient, there were significant losses of labeled HA in the subsequent ion-exchange step. Thus, this macromolecule was measured by an alternative procedure which involved chondroitinase digestion and analysis of the disaccharides derived from both the HA and the DS-CS PGs. The known specific activity of the radioisotope in the medium and the ratio of $^{3H}/[^35S]$ in the chondroitin 4-sulfate disaccharide were used to correct for changes in specific activity of hexosamine pools so that the amounts of HA and PGs synthesized/time could be estimated.

COCs stimulated to mucify by FSH in the presence of FCS synthesized HA at $\sim$10 times the rate for unstimulated COCs during 18 h of treatment, and most of this newly labeled HA accumulated in the COC matrix (Fig. 5). The time course for the stimulation of HA synthesis and its accumulation correlates with COC expansion. After FSH stimulation, HA synthesis and accumulation was four to five times the control in the first 0 h, increased to 20-30 times the control during 3-12 h, and decreased back to four to six times the control during 12-18 h (Fig. 7). COC expansion in these conditions becomes apparent 4-5 h after FSH stimulation and appears to reach completeness by morphological criteria by 12-14 h. The net increase in HA in the COC matrix during the 18 h was $\sim$2.0 ng/COC which would contribute a concentration of 250 $\mu$g of HA/ml in the matrix based on the estimated volume of the expanded COC (8 $\times$ 10$^{-3}$ mm$^3$). COCs stimulated by FSH in the absence of FCS increased net HA synthesis as much or more as for COCs stimulated by FSH in the presence of FCS. However, the HA accumulates in the medium and not the COC matrix (Fig. 9), and these COCs do not expand. These results confirm and extend those reported by Eppig (37) who used cetylpyridinium chloride precipitation of $^3H$-labeled macromolecules and treatment with Streptomyces hyaluronidase to show that HA was present in the COC matrix for cultures stimulated to mucify in the presence of FCS and in the medium when FCS was absent.

PGs bind to HA in some connective tissue matrices such as cartilage (17) and are important structural components in the organization of such matrices. PG synthesis and accumulation in the COC matrix did increase during mucification to levels two to three times the control values during the 18 h of treatment (Fig. 5). However, this increase does not appear to contribute significantly to the expansion or organization of the COC matrix since xyloside treatment effectively prevented the accumulation of PGs in the COC matrix (Table III) but did not prevent expansion. The PGs synthesized by the COC have molecular properties similar to those synthesized by rat ovarian granulosa cells. One HS PG, partially included on Superose 6, and two CS/DS PGs, one excluded and one partially included, were identified (Fig. 8). If the PGs are homologous, the observation that the PGs synthesized by mural granulosa cells do not bind to HA (39) would be consistent with the lack of their direct involvement in the organization of the HA matrix. However, PGs synthesized by cumulus cells may be an important component of the environment of the oocyte and for the fertilization process. It has been reported that the zona pellucida of isolated mouse
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2 A. Salustri, M. Yanagishita, and V. C. Hascall, unpublished observations.
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