Trimble, R. B., Maley, F., & Chu, F. K. (1983) *J. Biol. Chem. 258*, 2562-2567.
Ugalde, R. A., Staneloni, R. J., & Leloir, L. F. (1979) *Biochem. Biophys. Res. Commun. 91*, 1174-1181.
Ugalde, R. A., Staneloni, R. J., & Leloir, L. F. (1980) *Eur. J. Biochem. 113*, 97-103.
Wright, A., & Robbins, P. W. (1965) *Biochim. Biophys. Acta 104*, 594-596.

Estrogen Induces N-Linked Glycoprotein Expression by Immature Mouse Uterine Epithelial Cells†

Daniel D. Carson* and Jy-Ping Tang
Department of Biochemistry and Molecular Biology, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, Texas 77030

Received November 29, 1988; Revised Manuscript Received March 3, 1989

ABSTRACT: Characterization of complex glycoconjugates and the effects of estrogen on their expression in immature mouse uterine epithelial cells are reported. The secreted fraction contained nonanionic, O-linked lactosaminoglycan (LAG)-bearing proteins of M_r 30 000–40 000 as well as anionic, O-linked, LAG-bearing glycoproteins with very high apparent molecular weight (>670K). Heparan sulfate (HS) proteoglycans and HS linked to little or no protein were found in the secreted fraction as well. A very similar array of glycoconjugates was found in the nonhydrophobic fraction of cell-associated macromolecules. In addition, the hydrophobic cell-associated fraction contained nonanionic, LAG-bearing glycoproteins of approximately 250K, anionic LAG-bearing glycoproteins distributing over a wide range of molecular weights, and HS proteoglycans with median molecular weights of approximately 250K. In contrast to the glycoproteins produced by their mature counterparts, virtually all glycoproteins produced by immature cells were O-linked. Estrogen treatment of immature mice caused uterine epithelial cells to secrete anionic, high molecular weight (>670K) N-linked glycoproteins as a major product. These estrogen-responsive glycoproteins did not appear to contain LAGs. Estrogen treatment also markedly decreased the proportion of all hydrophobic glycoconjugates in the cell-associated fraction. Collectively, these observations indicate that one aspect of the estrogen-induced maturation of uterine epithelial cells is the stimulation of N-linked glycoprotein synthesis and secretion. Furthermore, stimulation of N-linked glycoprotein synthesis by itself is insufficient to support N-linked LAG glycoprotein production.

Alterations in glycoprotein expression have been associated with a variety of stimuli and developmental processes. A particularly interesting example of this is embryo implantation into the uterus. Both morphological (Lee et al., 1983) and biochemical (Farach et al., 1987) studies indicate that marked changes in the pattern of glycoconjugate expression occur at both embryonic and uterine cell surfaces during mouse embryo implantation. Furthermore, it appears that glycoconjugates directly participate in cell recognition/adhesion events that occur in this system (Farach et al., 1987; Dutt et al., 1987; Carson et al., 1987a). It has been well documented that expression of a uterine state that is permissive or receptive to embryo implantation is strictly controlled by steroid hormones (Psychoyos, 1973). Consequently, these hormones also should influence the expression of uterine glycoconjugates involved in embryo implantation. Uterine cells do not retain their steroid responsiveness in vitro (Glasser, 1985). Consequently, model systems for the study of uterine steroid hormone responses have been animals without endogenous sources of steroids, i.e., immature or castrate adult. Injection of these animals with particular steroids defines their hormonal status, and the subsequent responses can be studied in tissue explants or primary cultures. Using this approach in adult mice, it has been shown that estrogens markedly stimulate N-linked glycoprotein synthesis (Dutt et al., 1986, 1988) as well as dolichol-linked oligosaccharide assembly (Carson et al., 1987b) in uteri; however, it is not clear which uterine cell types are involved in this response. It also appears that estrogen has anabolic effects with regard to glycoprotein expression since turnover of heparan sulfate proteoglycans of uterine epithelial cells is stimulated by estrogen (Morris et al., 1988). Thus, estrogens exert their effects on uterine glycoprotein expression at a number of levels.

One interesting class of uterine N-linked glycoproteins are those bearing lactosaminoglycans (LAGs).[1] These polysaccharides have been detected through the use of the LAG-degrading enzyme endo-β-galactosidase (Fukuda, 1985) and the LAG-specific lectin pokeweed mitogen (Irimura & Nicolson, 1983). Uterine LAG synthesis in ovariectomized mature mice is preferentially stimulated by estrogens (Dutt et al., 1988). Furthermore, it has been shown that LAGs are major cell surface polysaccharides of uterine epithelial cells where they participate in aspects of cell adhesion (Dutt et al., 1987). Heparan sulfate proteoglycans (HSPGs) also are expressed at the cell surface of both the uterine epithelium and periimplantation-stage blastocysts (Farach et al., 1987; Tang et al., 1987). The blastocyst HSPGs appear to participate in

† This work was supported by grants from the National Institutes of Health (HD-18768) and the March of Dimes (1-958) awarded to D.D.C.
* To whom correspondence should be addressed.

[1] Abbreviations: E_2, 17β-estradiol; HS, heparan sulfate; LAG, lactosaminoglycan.

0006-2960/89/0428-8116$01.50/0

the initial phases of embryo attachment to a variety of substrates including uterine epithelial cells (Farach et al., 1987). Moreover, the epithelial cells express complementary HS-binding proteins at their cell surfaces that can support embryo attachment.[2] Thus, it appears that these complex glycoproteins not only are hormonally responsive but also participate in a number of aspects of uterine epithelial cell–cell interactions.

Previous studies have shown that immature rat uterine epithelial cells synthesize O-linked, linear, and more highly sulfated LAG-bearing glycoproteins than uterine epithelial cells from mature mice (Carson et al., 1988); however, both cell types express similar HSPGs. Possible explanations for the differences in LAG expression include species-specificity, extracellular matrix influences, and/or maturational or developmental changes. In the present studies, we have utilized primary cultures of uterine epithelial cells derived from immature mice to study factors influencing glycoprotein expression. As a result, we have isolated and characterized a number of the major, intact glycoconjugates expressed by these cells. Our observations demonstrate that immature uterine epithelial cells synthesize similar HSPGs as mature cells; however, immature uterine epithelial cells express very low amounts of N-linked oligosaccharides. Instead, these cells express O-linked, more highly sulfated oligosaccharides, including LAGs. Thus, immature uterine epithelial cells from both rats and mice are similar with regard to their expression of O-linked, sulfated LAGs. These immature LAGs markedly differ from mature LAG structures (Dutt et al., 1988). Estrogen treatment of immature mice stimulates production and secretion of high molecular weight, N-linked glycoproteins; however, these N-linked structures do not include LAGs. These data demonstrate a marked effect of estrogen on N-linked glycoprotein expression in a particular cell type of immature uteri, the epithelial cells. It is suggested that one aspect of the development of a mature phenotype in uterine epithelia is the estrogen-induced expression of N-linked glycoproteins; however, additional maturational factors are required for N-linked LAG expression.

Materials and Methods

Materials. CF-1 mice were obtained from Harlan/Sprague-Dawley (Houston, TX). $H_2{}^{35}SO_4$ (carrier-free) and [6-^{3}H]glucosamine (25 Ci/mmol) were purchased from ICN Biomedicals, Inc. (Costa Mesa, CA). Tissue culture media and supplements were from Irvine Scientific (Santa Ana, CA). Tissue culture plates were from Falcon Division, Becton-Dickinson and Co. (Oxnard, CA). Urea and guanidine hydrochloride were purchased from Schwarz/Mann Biotech (Cleveland, OH). Octyl β-D-glucopyranoside (octyl glucoside), octyl-Sepharose, Triton X-100, CHAPS, leupeptin, antipain, benzamidine, aprotinin, chymostatin, Pronase, chondroitinase ABC, Jack bean β-*N*-acetylhexosaminidase, *Escherichia coli* β-galactosidase, Jack bean α-mannosidase, cycloheximide, pepstatin, keratan sulfate, pokeweed mitogen–agarose, and 17β-estradiol were from Sigma Chemical Co. (St. Louis, MO). *Clostridium perfringens* neuraminidase was from Worthington Diagnostic Systems, Inc. (Freehold, NJ). Endo-β-galactosidase (*Pseudomonas* keratanase), keratan sulfate, endo-β-*N*-acetylhexosaminidase H, heparitinase, and heparan sulfate were purchased from Miles Scientific (Naperville, IL). Peptide:*N*-glycanase was purchased from Genzyme (Boston, MA). Protein molecular weight calibration standards were from Bethesda Research Laboratories (Gaithersburg, MD). All chemicals used were reagent grade.

Metabolic Labeling. Uterine epithelial cell cultures were prepared from immature (14–18 days old) mice exactly as described (Dutt et al., 1986). To test the effects of estrogen, immature mice were injected on each of 3 consecutive days with 20 ng of 17β-estradiol and were used on the day following the third injection for preparation of cell cultures. Previous studies (Dutt et al., 1986) have described the dose response of uterine glycoprotein synthesis to estrogen. The dose of estrogen used in the present studies was adjusted to compensate for the lower (one-fifth) body weight of immature mice. Cultures were established for 24 h prior to use for metabolic labeling experiments. Metabolic labeling was performed overnight (16–18 h) in a humidified atmosphere of air/CO_2, 95:5 (v/v), at 37 °C in serum-free medium containing 0.5 mCi/mL $H_2{}^{35}SO_4$ and 100 μCi/mL [6-^{3}H]glucosamine. Preliminary experiments demonstrated that labeling of the cell-associated material reached equilibrium by 8–10 h. The medium used was RPMI 1640 (minus sulfate) supplemented with 3.3 mM $MgCl_2$, 1.2 g/L $NaHCO_3$, 15 mM Hepes (pH 7.2), 2.5 units of penicillin/mL, and 2.5 μg of streptomycin sulfate/mL. The streptomycin sulfate served as the sole source of nonradioactive sulfate in these experiments. Previous studies have shown that glycosaminoglycan assembly occurs normally in the low-sulfate medium used (Tang et al., 1987). In some experiments, cells were labeled in the presence of 2.8 μg/mL cycloheximide. In these studies, the inhibitor was added at the same time as the isotope and the incubation continued for 5 h prior to harvest. Parallel control cultures were incubated for the same period of time in medium not supplemented with cycloheximide.

Analyses of Glycoconjugates. Macromolecular material in the secreted and cell-associated fractions was isolated under dissociative conditions exactly as described previously (Tang et al., 1987). The components in the cell-associated extracts were first fractionated by hydrophobic affinity chromatography on octyl-Sepharose (Tang et al., 1987). Briefly, the extracts were dissolved in 3 mL of a solution of 4 M guanidine/20 mM Tris–acetate (pH 7.0) and incubated batchwise with 0.5 mL of octyl-Sepharose (approximately a 50% suspension) for 1 h at room temperature with constant rotary agitation. Subsequent elutions were performed using the same guanidine-containing buffer supplemented with 0.1%, 0.5%, or 1% Triton X-100 (v/v) as indicated. At each elution step, the resin was washed 3 times with 1 mL of the indicated solution before proceeding to the next elution step. Recoveries of radioactivity from this procedure exceeded 85% in all cases. These fractions were dialyzed against water before further use.

Various fractions of the cell-associated material as well as the secreted material were subjected to anion-exchange liquid chromatography as described previously (Tang et al., 1987). The liquid chromatographic system was purchased from Beckman Instruments (Berkeley, CA) and consisted of two Model 100A pumps controlled by a Model 421A controller. Absorbance was monitored at 280 nm using an on-line Model 163 variable-wavelength detector interfaced with a Model 427 integrator. Chromatography was performed on a 0.5 × 5 cm column of Mono Q (Pharmacia Fine Chemicals; Uppsala, Sweden), equilibrated with 0.5 M urea, 20 mM Tris–acetate (pH 7.0), 0.01% (w/v) octyl glucoside, and 0.02% (w/v) sodium azide. The gradient was developed with 0–4 M sodium chloride as indicated on the graphs. The column was pumped at a flow rate of 1 mL/min. Fractions were collected every 0.5 min. Recoveries of radioactivity from these analyses typically ranged from 85–95%.

[2] O. Wilson, S. Stewart, and D. D. Carson, submitted for publication.

Macromolecules also were analyzed by molecular exclusion liquid chromatography using a 1 × 30 cm column of Superose 12 (Pharmacia Fine Chemicals, Uppsala, Sweden). These columns were equilibrated with 2 M guanidine, 20 mM Tris–acetate (pH 7.0), 0.01g (w/v) octyl glucoside, and 0.02% (w/v) sodium azide and eluted at a flow rate of 0.7 mL/min. Fractions were collected every 0.5 min. Recoveries of radioactivity from this procedure exceeded 85%.

Enzymatic and Chemical Degradation of Glycoconjugates. Heparitinase digestions (200 milliunits/mL) were performed overnight at room temperature in 50 mM Tris–acetate (pH 7.0), 5 mM $CaCl_2$, 100 mM NaCl, 100 μg/mL bovine serum albumin, and a mixture of protease inhibitors (6). Jack bean α-mannosidase digestions (20 milliunits/mL) were performed for 16 h at 25 °C in 0.1 M citrate (pH 5.0). Neuraminidase (*Clostridium perfringens*; 0.5 unit/mL) digestions were performed for 16 h at 25 °C in 20 mM sodium acetate (pH 5.0). β-Galactosidase (*E. coli*; 0.3 unit/mL) digestions were performed at 25 °C for 16 h in 20 mM Tris–acetate (pH 7.5), 10 mM $MgCl_2$, 10 mM β-mercaptoethanol, and 10 mM NaCl. β-*N*-Acetylhexosaminidase (Jack bean; 20 milliunits/mL) digestions were performed at 25 °C for 16 h in 0.1 M sodium citrate (pH 4.0). Enzyme activity was monitored in parallel incubations using the appropriate *p*-nitrophenyl glycosides. Peptide:*N*-glycanase (10 units/mL) digestions were performed at 25 °C for 16 h in 150 mM sodium phosphate (pH 8.5), 60 mM EDTA, 1% (v/v) β-mercaptoethanol, 1% (v/v) Triton X-100, and 2% (w/v) sodium dodecyl sulfate. Endo-β-*N*-acetylglucosaminidase H (30 milliunits/mL) digestions were performed at 25 °C for 16 h in 20 mM tris-HCl (pH 7.2), 0.2% (w/v) sodium dodecyl sulfate, and 0.2% (v/v) β-mercaptoethanol. Endo-β-galactosidase digestions were performed at 25 °C for 48 h in 0.1 M Tris-HCl (pH 7.2) containing 1.5 units/mL endo-β-galactosidase. Fresh enzyme was added after 24 h. To ensure that the enzymes were active, parallel digestions with heparitinase or endo-β-galactosidase always were performed using 1 mg/mL heparan sulfate or keratan sulfate, respectively. Depolymerization of the standard glycosaminoglycans was monitored by cetylpyridinium chloride precipitation (Dutt et al., 1987). Mild alkaline hydrolysis (β-elimination) was performed for 48 h at 37 °C in 0.1 M NaOH and 0.25 M sodium borohydride. Pronase digestions were performed exactly as described previously (Dutt et al., 1986).

Lectin-Binding Studies. Pokeweed mitogen binding was monitored by incubating ^{3}H-labeled glycoconjugates in 0.5 mL of phosphate-buffered saline (PBS) containing 0.1% (w/v) octyl glucoside and 0.1% (w/v) bovine serum albumin with 40 μL of pokeweed mitogen–agarose (approximately a 50% suspension). These suspensions were incubated for 1 h at room temperature with constant rotary agitation. The unbound material was removed by centrifugation followed by three washes with 1 mL of the binding buffer. The radioactivity associated with the resin was determined by resuspending the pellet in PBS and transferring it to scintillation vials for counting. Recoveries from this procedure ranged from 85 to 95%. Previous studies have demonstrated the specificity of binding to the lectin under these conditions (Dutt et al., 1988).

Results

Identification of Glycoconjugates Secreted by Immature Epithelial Cells. In order to identify the glycoconjguates produced by immature uterine epithelial cells, these cells were labeled metabolically with [^{3}H]glucosamine and $H_2{}^{35}SO_4$ for 16–18 h. The radioactive, secreted macromolecules were obtained from the culture medium by desalting and then fractionated by anion-exchange liquid chromatography. As shown in Figure 1, three major charge classes were obtained. These were (1) components that failed to bind to the resin, (2) components that bound and eluted with 0.9–1.2 M NaCl, and (3) components that bound and eluted with 2.5–4 M NaCl. The components in each of these charge classes were studied further using a series of specific enzymatic and chemical digestions followed by analyses of the products by molecular exclusion liquid chromatography.

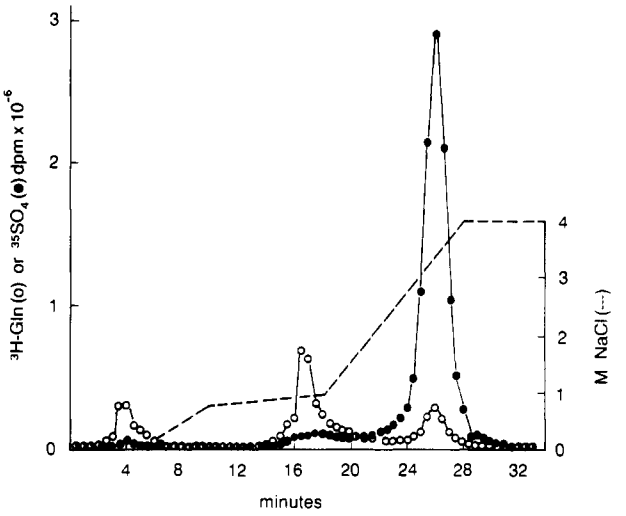

FIGURE 1: Anion-exchange chromatography of glycoconjugates secreted by immature uterine epithelial cells. Primary cultures of immature mouse uterine epithelial cells were metabolically labeled overnight with [^{3}H]glucosamine and $H_2{}^{35}SO_4$ and macromolecules isolated as described under Materials and Methods. This material was chromatographed on a 0.5 × 5 cm Mono Q column eluted as described under Materials and Methods and fractions were collected every 0.5 min. The sodium chloride gradient (---) is indicated. Typical elution profiles of ^{3}H-labeled (○) or $^{35}SO_4$-labeled (●) macromolecules are shown.

The intact components in the unbound fraction from the anion-exchange resin were labeled primarily with ^{3}H (see Figure 1); however, in all cases, the behavior of the sulfated components in this fraction was the same as that of the ^{3}H-labeled components (data not shown). As shown in Figure 2A, the intact components in this fraction migrated as a single major peak of radioactivity and exhibited median hydrodynamic radii of a 35-kDa protein. Pronase digestion of this material shifted the distribution of radioactivity to a median corresponding to the hydrodynamic radius of a 18-kDa protein, indicating that most of these components were linked to protein. Digestion with endo-β-galactosidase converted about 20% of the radioactivity to a much lower molecular weight form, indicating that many of the polysaccharides in this fraction contained GlcNAc(β→)Gal sequences. Very little material in this fraction bound to pokeweed mitogen (Table I). As shown in Figure 3A, mild alkaline hydrolysis of this material quantitatively released the radioactivity to a much lower molecular weight form, demonstrating that almost all of these oligosaccharides were O-linked. Collectively, these data indicated that the components in this fraction were primarily composed of O-linked glycoproteins; many of these glycoproteins expressed LAG sequences.

The intact components of the 0.9–1.2 M eluate from the anion-exchange column not only were more highly negatively charged than the components in the unbound fraction but also were much larger. As shown in Figure 2B, these components migrated near the fully excluded column volume of Superose 12, indicating that they had hydrodynamic radii greater than that of a 670-kDa protein. Pronase digestion of this material

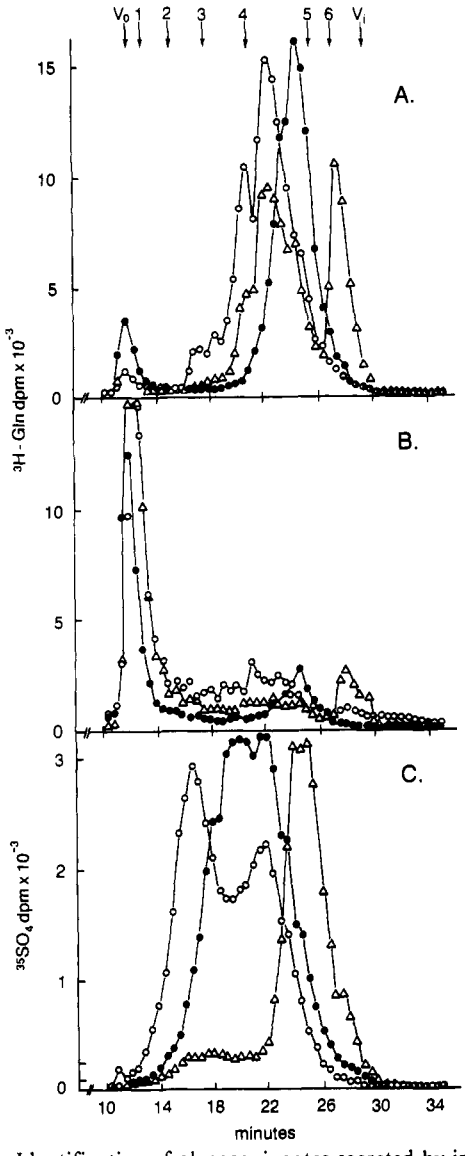

FIGURE 2: Identification of glycoconjugates secreted by immature uterine epithelial cells. Secreted glycoconjugates that eluted from anion-exchange liquid chromatography in the run-through (panel A) and 0.9–1.2 M NaCl (panel B) or 2.5–4 M NaCl (panel C) eluates were chromatographed on a 1 × 30 cm column of Superose 12 as described under Materials and Methods. Fractions were collected every 0.5 min. Representative profiles of the intact material (○), the Pronase-digested material (●), and the endo-β-galactosidase-digested (△; panels A and B) or heparitinase-digested (▲; panel C) material are shown. For simplicity, only the profiles of the ^{3}H-labeled components are shown in panels A and B, while those of the $^{35}SO_4$-labeled components are shown in panel C. The elution positions of the following molecular weight markers are indicated at the top of the figure: V_0, Blue Dextran (M_r 2 × 10^6); 1, thryoglobulin; 2, catalase; 3, rabbit IgG; 4, ovalbumin; 5, cytochrome *c*; 6, aprotinin; V_i, potassium dichromate.

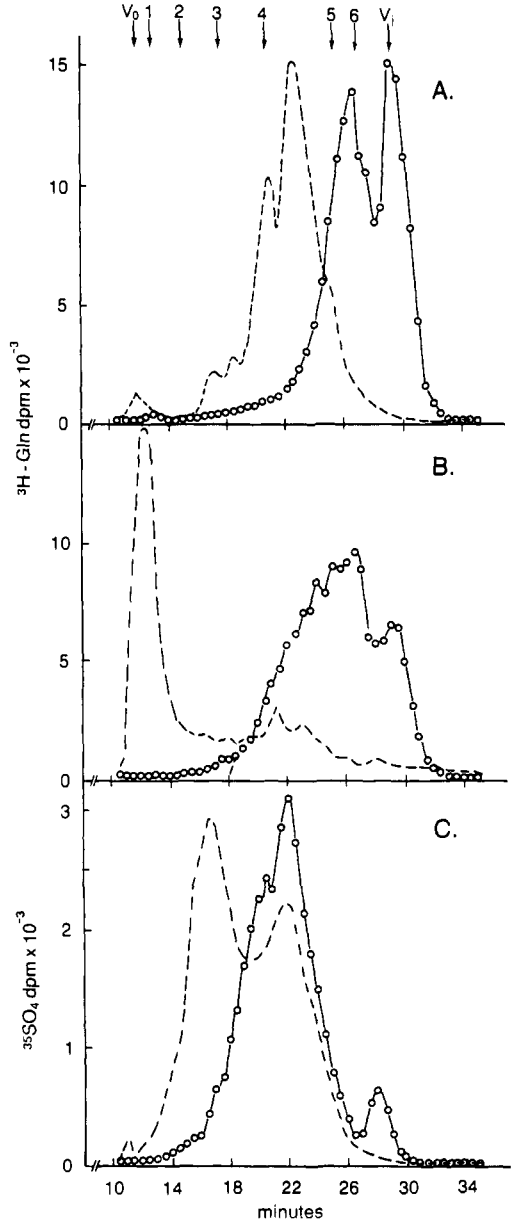

FIGURE 3: Mild alkaline hydrolysis of glycoconjugates secreted by immature uterine epithelial cells. Secreted glycoconjugates were analyzed by Superose 12 chromatography before (---) or after (○) they had been subjected to mild alkaline hydrolysis as described under Materials and Methods. Representative profiles are shown of ^{3}H-labeled glycoconjugates from the run-through (panel A) and 0.9–1.2 M NaCl (panel B) eluates and $^{35}SO_4$-labeled glycoconjugates from the 2.5–4 M NaCl eluates (panel C) from anion-exchange liquid chromatography (see Figure 1). The molecular weight markers indicated at the top of the figure are the same as described in Figure 2.

released a small fraction of the radioactivity to a form that eluted with the characteristics of an 18-kDa protein. In addition, endo-β-galactosidase digestion released a small portion (11%) of the radioactivity to a form that eluted near the fully included column volume. Only a small percentage of radioactivity bound to pokeweed mitogen (Table I). Again, mild alkaline hydrolysis of this fraction quantitatively converted the radioactivity to lower molecular weight forms with a size distribution ranging from 5K to 100K relative to protein standards (Figure 3B). It was concluded that most of the ^{3}H-labeled components in this fraction were composed of high molecular weight glycoproteins containing O-linked oligosaccharides, including LAGs.

The behavior of the majority of the sulfated components in this fraction was different from that of the major ^{3}H-labeled components. As shown in Figure 4A, most of the sulfated components displayed similar size characteristics as the major ^{3}H-labeled molecules from the unbound fraction from the anion-exchange resin. They also were similar with regard to Pronase and mild alkali susceptibility. A much larger portion (34%) of the sulfated than the ^{3}H-labeled molecules in this fraction were converted to low molecular weight forms by endo-β-galactosidase. It was concluded that the sulfated molecules in this fraction were, for the most part, distinct from the majority of the ^{3}H-labeled components. These components resembled the glycoproteins from the unbound fraction from the anion-exchange resin and included sulfated LAGs.

Table I: Characteristics of Major [^{3}H]Glucosamine-Labeled Glycoconjugates from Immature Uterine Epithelial Cells[a]

fraction	% released with endo-β-galactosidase	% binding to pokeweed mitogen
cell-associated		
$-E_2$ A	20 ± 1	16 ± 1
$-E_2$ B	22 ± 4	15 ± 1
$+E_2$ A	<1	24 ± 1
$+E_2$ B	7 ± 2	24 ± 5
secreted		
$-E_2$ A	20 ± 10	3 ± 1
$-E_2$ B	11 ± 1	4 ± 1
$+E_2$ A	22 ± 2	14 ± 1
$+E_2$ B	5 ± 3	1 ± 1

[a] [^{3}H]Glucosamine-labeled glycoconjugates were obtained from the cell-associated or secreted fractions of primary cultures of uterine epithelial cells and subfractionated by anion-exchange liquid chromatography as described in Figure 1. The material eluting in the run-through is designated A, and the material eluting with 0.9–1.2 M NaCl is designated B in this table. Portions of these subfractons were digested with endo-β-galactosidase as described under Materials and Methods. The percentage of radioactivity released to a form that migrated near the fully included volume of Superose 12 (see Figure 2) then was determined. Other portions were analyzed by lectin affinity chromatography using pokeweed mitogen–agarose as described under Materials and Methods. Values represent the averages ± range of values obtained for at least two determinations performed for samples derived from at least two cell cultures. In the instances where only one value is presented, it represents the data obtained for a single determination. $+E_2$ represents cells from immature mice that had received 20 ng of 17β-estradiol on each of the 3 days prior to preparation of the cell cultures.

In contrast to the other secreted glycoconjugates, the intact components of the high-salt (2.5–4 M NaCl) eluate were primarily labeled with $H_2{}^{35}SO_4$. The behavior of the ^{3}H-labeled molecules of this fraction was similar to that described below for the sulfated components. The intact molecules eluted from the molecular exclusion column in two broad, major peaks (Figure 2C). The median hydrodynamic radii of these peaks corresponded to those of proteins of 230 and 40 kDa, respectively. Pronase digestion shifted the size distribution of the larger components of this fraction but did not generate any forms smaller than the lower molecular weight components from the undigested material. Most (75%) of the radioactivity in either size class was susceptible to digestion with heparitinase, indicating that both size classes were largely composed of heparan sulfate containing molecules. Mild alkaline hydrolysis of this fraction generated one major, broad peak of radioactivity with a median size similar to that of a 40-kDa protein (Figure 3C). It was concluded that the higher molecular weight components of this fraction consisted primarily of heparan sulfate proteoglycans while the lower molecular weight components consisted primarily of heparan sulfate polysaccharides linked to little or no protein.

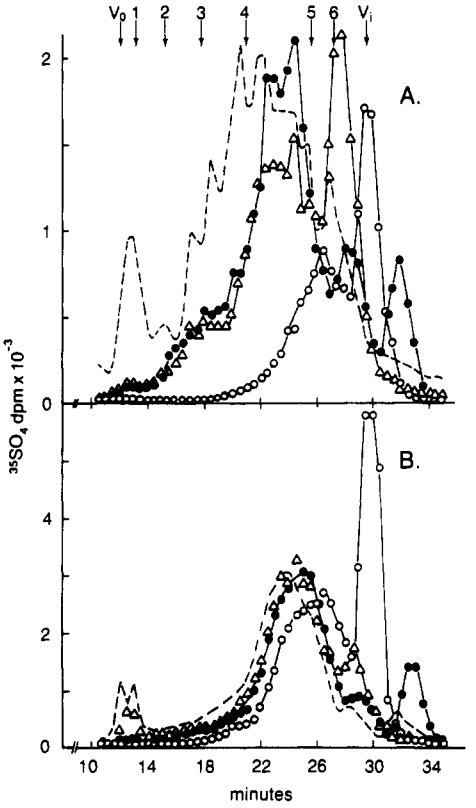

FIGURE 4: Molecular exclusion chromatography of sulfated components in 0.9–1.2 M NaCl eluates from Mono Q. The elution profiles of the sulfated components obtained from either secreted (panel A) or cell-associated glycoconjugates (panel B) eluting from anion-exchange liquid chromatography with 0.9–1.2 M NaCl are shown. The nonhydrophobic, cell-associated glycoconjugates, i.e., run-through fractions from octyl-Sepharose, were used for these analyses. Each panel shows representative elution profiles obtained for intact material (- - -), Pronase-digested material (●), endo-β-galactosidase-digested material (△), or material subjected to mild alkaline hydrolysis (○) as described under Materials and Methods. For comparison to the ^{3}H-labeled components on these fractions, see Figures 2B and 3B (secreted) and Figures 6B and 7B (cell-associated). The molecular weight markers indicated at the top of the figure are the same as described in Figure 2.

Identification of Cell-Associated Glycoconjugates from Immature Epithelial Cells. The metabolically labeled, cell-associated glycoconjugates were extracted with guanidine and detergent in the presence of protease inhibitors and the macromolecular components obtained by desalting. In preliminary experiments it was found that cycloheximide treatment inhibited both [^{3}H]glucosamine and $H^{35}SO_4$ incorporation into the cell-associated fraction by 75–90%, indicating that most of these components were linked to or derived from proteins (data not shown). The material in the cell-associated fractions was separated first by hydrophobic affinity chro-

Table II: Hydrophobic Affinity Chromatography of Cell-Associated Glycoconjugates

cell source	precursor	% radioact. eluting with indicated Triton X-100 concn[a] 0%	0.1%	0.5%	1.0%	>1.0%
immature	[^{3}H]Gln	56 ± 1	11 ± 1	25 ± 1	7 ± 1	2 ± 1
immature + E_2		92 ± 1	1 ± 1	5 ± 1	1 ± 1	1 ± 1
immature	$^{35}SO_4$	78 ± 4	6 ± 2	11 ± 2	3 ± 1	1 ± 1
immature + E_2		95 ± 1	1 ± 1	1 ± 1	1 ± 1	1 ± 1

[a] Primary cultures of uterine epithelial cells were prepared from immature mice and metabolically labeled overnight with [^{3}H]glucosamine and $H_2{}^{35}SO_4$ as described under Materials and Methods. Immature + E_2 represents immature mice that were treated with 20 ng of 17β-estradiol on each of 2 consecutive days immediately prior to preparation of the primary cultures. Macromolecules in the cell-associated fraction were extracted and desalted as described previously (Tang et al., 1987). This material was fractionated by batchwise elution from octyl-Sepharose as described under Materials and Methods. Recoveries of radioactivity from these analyses ranged between 85 and 95% for both precursors. The data show the averages (± variation) of separate determinations performed on two or more cultures. The values presented are the percentages of radioactivity recovered from the octyl-Sepharose resin using the indicated concentration of Triton X-100 in the eluting buffer.

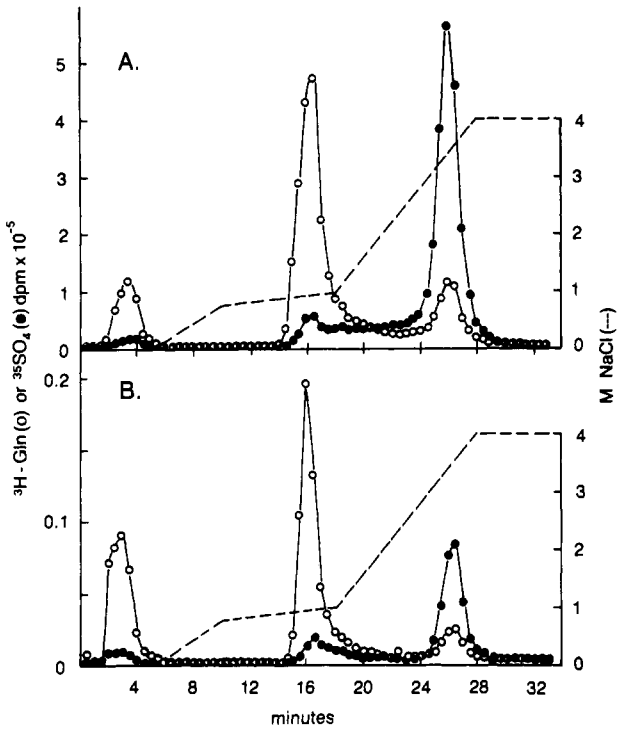

FIGURE 5: Anion-exchange liquid chromatography of cell-associated glycoconjugates from immature uterine epithelial cells. The metabolically labeled, cell-associated glycoconjugates extracted from primary cultures of uterine epithelial cells derived from immature mice were first fractionated by octyl-Sepharose chromatography as described in Table I. The materials eluting in the run-through (nonhydrophobic; panel A) or the 0.5% (v/v) Triton X-100 (panel B) eluates then were analyzed by anion-exchange liquid chromatography as described in Figure 1. The NaCl gradient used is shown (---). Representative profiles of the ^{3}H-labeled (○) and $^{35}SO_4$-labeled (●) components are shown in either case.

matography on octyl-Sepharose under dissociative conditions. As shown in Table II, most of the radioactivity (^{3}H or $^{35}SO_4$) did not bind to this resin, suggesting that these components lacked significant hydrophobic character. The largest proportion of radioactivity in the bound material was eluted with 0.5% Triton X-100. The materials in the unbound (nonhydrophobic) and the 0.5% Triton X-100 eluate (hydrophobic) were chosen for further study.

As shown in Figure 5A, the radioactive components in the nonhydrophobic fraction yielded three major charge classes following anion-exchange liquid chromatography. The components in each of these charge classes were examined by the same series of chemical and enzymatic digestions described above for the secreted components. The characteristics of these cell-associated components were virtually identical with those of the corresponding charge class of secreted components, e.g., size, sensitivity to mild alkaline hydrolysis, etc. (data not shown). The hydrophobic, cell-associated components (0.5% Triton X-100 eluate from octyl-Sepharose) differed by virtue of their hydrophobic character. In addition, the nonanionic fraction primarily consisted of ^{3}H-labeled components with a median molecular weight similar to that of a 250-kDa protein; however, by all other criteria applied, i.e., enzymatic and chemical stability, the major components in each charge class behaved similarly as the corresponding charge class in the secreted fraction (data not shown). To summarize these studies, the unbound radiolabeled material from Mono Q was primarily composed of O-linked glycoconjugates, including lactosaminoglycans. This material had a greater molecular weight (~250K) in the hydrophobic, cell-associated fraction than the corresponding nonhydrophobic, cell-associated or secreted fraction (M_r ~30K–40K). The radiolabeled material eluting with 0.9–1.0 M NaCl consisted of high molecular weight O-linked glycoconjugates, including lactosaminoglycans. The radiolabeled material eluting with 2.5–4.0 M NaCl primarily consisted of heparan sulfate polysaccharides and proteoglycans. The hydrophobic material in this charge class appeared to exclusively contain proteoglycans with a median molecular weight of 250K relative to protein standards. Most of the cellular oligosaccharides were protein-linked. In all cases, greater than 90% of these oligosaccharides were of the O-linked variety.

Factors Influencing Glycoprotein Expression by Immature Epithelial Cells. The major glycoconjugates expressed by immature mouse uterine epithelial cells differed from those expressed by mature cells (Dutt et al., 1988) in three major respects. First and foremost, almost all of the ^{3}H-labeled molecules were in O-linkage to protein, i.e., alkali-labile. Second, many of the LAG structures were sulfated. Third, the immature LAGs both were more susceptible to endo-β-galactosidase and bound less well to pokeweed mitogen than their mature counterparts. These observations suggested that the immature LAG structures were more linear and/or less substituted than the mature structures (Fukuda, 1985). Analyses of the glycoproteins produced by immature rat uterine epithelial cells cultured on a basement membrane like extracellular matrix indicated a predominance of linear, O-linked, highly sulfated LAGs in this case, as well (Carson et al., 1988). Therefore, it was of interest to determine what factors contributed to the differences in glycoprotein expression between immature and mature mouse uterine epithelial cells. Analyses of the patterns of glycoconjugates produced by immature cells cultured on a basal lamina-like extracellular matrix (Matrigel) revealed no differences in the patterns of glycoprotein expression compared to cells not cultured on these matrices. These parameters included chromatographic characteristics as well as sensitivity to Pronase, endo-β-galactosidase, and alkali lability (data not shown). Thus, it did not appear that exposure to basal lamina could account for the differences between the mature and immature phenotypes.

Estrogen has been shown to markedly stimulate uterine synthesis of N-linked glycoproteins, in general (Dutt et al., 1986), and N-linked LAGs, in particular (Dutt et al., 1988). Consequently, it was considered that the estrogen exposure that occurs during uterine maturation might stimulate N-linked glycoprotein production by uterine epithelial cells. To test this hypothesis, epithelial cells form estrogen-treated, immature mice were labeled metabolically with [^{3}H]glucosamine and $H_2{}^{35}SO_4$ to monitor effects on glycoprotein synthesis. The characteristics of the intact glycoconjugates produced by these cells were monitored exactly as described above for cells from immature mice not receiving estrogen.

No differences in the ion-exchange profiles of the secreted and cell-associated radiolabeled macromolecules, including LAGs, were observed. However, in the cell-associated fraction, less than 10% of the glycoconjugates bound to the hydrophobic affinity resin using either precursor as a monitor (Table II). Consequently, one action of estrogen appeared to be to decrease the expression of hydrophobic glycoconjugates.

Almost all of the major glyconconjugates produced by estrogen-treated cells exhibited the same characteristics as those described for non-estrogen-treated cells. The singular exception was the secreted components eluting from the anion-exchange resin with 0.9–1.2 M NaCl. This fraction constituted 50% of the secreted, [^{3}H]glucosamine-labeled macromolecules.

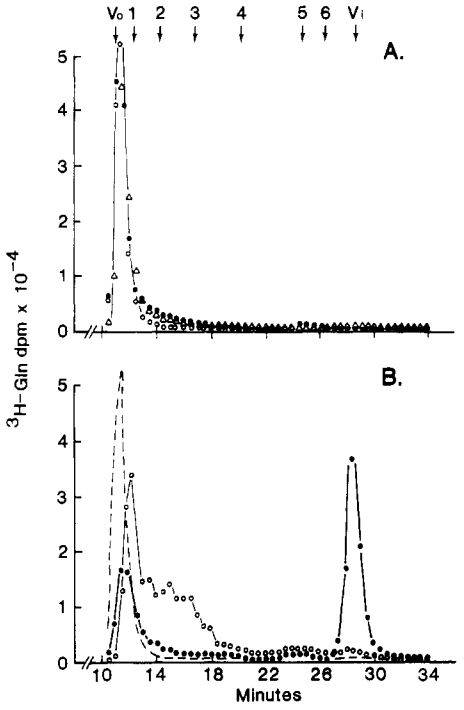

FIGURE 6: Identification of estrogen-induced, N-linked glycoproteins from immature epithelial cells. Materials from the 0.9–1.2 M NaCl eluate from the anion-exchange resin from the secreted fractions derived from estrogen-treated immature mice were analyzed by Superose 12 chromatography as described under Materials and Methods. The elution profiles of the intact (○), Pronase-digested (●), and endo-β-galactosidase-digested (△) material are shown in panel A. Panel B shows the elution profiles of the intact (---), mild alkali-treated (○), and peptide:*N*-glycanase-digested (●) material.

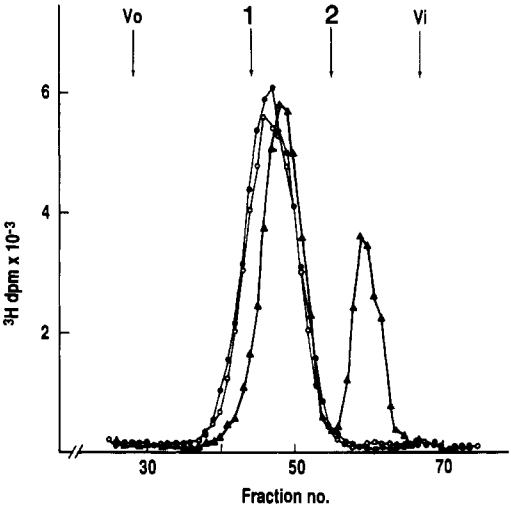

FIGURE 7: Sephadex G-50 chromatography of oligosaccharides released by peptide:*N*-glycanase. Oligosaccharides released from secreted glycoproteins from estrogen-treated immature cells such as described in Figure 6B were analyzed by Sephadex G-50 chromatography before (○) and after (●) digestion with Jack bean α-mannosidase or *Clostridium perfringens* neuraminidase (▲). The column (1.5 × 84 cm) was equilibrated with 0.1 M sodium bicarbonate, 5% (v/v) ethanol, and 0.03% (w/v) sodium azide. The elution positions of blue dextran (V_0), potassium dichromate (V_i), and fetuin glycopeptides 1 (M_r 3300) and 2 (M_r 1500) are indicated.

As shown in Figure 6A, this material eluted in the void volume of Superose 12 even after Pronase digestion and exhibited little sensitivity to endo-β-galactosidase or Pronase; however, [^{3}H]glucosamine incorporation into this material was inhibited approximately 80% by cycloheximide (data not shown). Consequently, it appeared that this material was protein-linked and that these oligosaccharides protected the protein cores from proteolytic attack. Very little of this material bound to pokeweed mitogen (Table II). These data indicated a paucity of LAG structures.

The most striking difference was that these secreted glycoproteins were much more resistant to mild alkaline hydrolysis than the similar fraction from non-estrogen-treated cells (Figure 6B). Furthermore, a large fraction (30–50%) of the radioactivity was released following digestion with peptide:*N*-glycanase. In contrast, no radioactivity was released by digestion with endo-β-acetylhexosaminidase H (data not shown). The low molecular weight oligosaccharides released by peptide:*N*-glycanase were characterized further. Only a small fraction, i.e., 6–7%, bound to concanavalin A–agarose. Molecular exclusion chromatography on Sephadex G-50 demonstrated that these oligosaccharides eluted as a single, symmetrical peak with a median molecular weight similar to that of the disialoglycopeptides of fetuin (Figure 7). The size distribution of these oligosaccharides was unaffected by digestion with α-mannosidase, indicating a lack of α-linked mannose residues at the nonreducing termini; however, digestion of these glycopeptides with neuraminidase generated two distinct peaks of radioactivity of lower molecular weight. The smaller product contained about 30% of the radioactivity, migrated near V_i and was assumed to contain the released sialic acids. The larger product contained about 70% of the radioactivity and could be reduced further in size by digestion with β-galactosidase and β-*N*-acetylhexosaminidase (data not shown). It was concluded that many of the oligosaccharides released by peptide:*N*-glycanase were complex, sialic acid containing N-linked structures. Production of these N-linked oligosaccharides was strongly dependent upon estrogen treatment.

Discussion

The pattern of glycoconjugates expressed at the cell surface of the uterine epithelium undergoes marked changes in response to steroid hormones and in preparation for embryo implantation. Studies of estrogen influences in uteri of mature mice have shown that this hormone has a strong stimulatory effect on N-linked glycoprotein assembly (Dutt et al., 1982, 1986); however, these studies did not demonstrate whether the epithelial cells of the uterus were responsive in this regard. These observations are further complicated by the use of mature, ovariectomized mice because such animals have been previously exposed to steroid hormones. Furthermore, these animals continue to be influenced by nonovarian hormones as well as other factors expressed by adult animals that may influence uterine physiology, e.g., peptide hormones. This combination of both influences may contribute to expression of a more differentiated phenotype and modulate the actions of estrogen on these cells (Quarmby & Korach, 1984; Maier et al., 1985).

In order to assess the effects of estrogen on glycoprotein expression by uterine epithelial cells in a simpler setting, we have utilized immature mice, i.e., prior to the onset of expression of maturational factors by the animal. Furthermore, we have focused on a particular uterine cell type, the epithelium, for these studies. A disadvantage of this focus is that it takes almost 2 days from the time the cells are removed from the uterus until the profiles of their metabolically labeled glycoproteins can be examined. Consequently, it is possible that some alterations in the patterns of expression may occur during this interval. Nonetheless, it appears that similar glycoconjugates are expressed by uterine epithelia both in vivo and in vitro (Dutt et al., 1987, 1988; Morris et al., 1988; Tang

et al., 1987), suggesting that drastic alterations do not occur. The present results indicate that estrogen alone can stimulate N-linked glycoprotein production by these cells. These observations support the previous contention that estrogen acts at a fundamental level to support N-linked glycoprotein assembly in this system (Dutt et al., 1986; Carson et al., 1987b). Nonetheless, estrogen alone is not sufficient to generate a mature phenotype with regard to the overall pattern of glycoprotein expression, e.g., N-linked LAG expression (Dutt et al., 1988). Consequently, it appears that other maturational influences must act in conjunction with estrogen to redirect epithelial LAG assembly. Such redirection may reflect expression of particular protein acceptors and/or modulation of particular glycosyltransferase activities. Hormonal effects on both types of processes have been described in mouse uteri (Dutt et al., 1986; Teng et al., 1986). In regard to estrogen actions on glycosyltransferases, it appears that some modulation of LAG assembly must occur, since several structural characteristics differ between the LAG polymers produced by immature and mature cells. These characteristics include an increased degree of sulfation and susceptibility to endo-β-galactosidase, as well as a decreased affinity for pokeweed mitogen (Dutt et al., 1988).

Throughout these studies, we considered that molecular weight shifts caused by alkaline hydrolyses might reflect peptide bond breakage rather than oligosaccharide release as seems to occur to a limited extent under these conditions (Plantner & Carlson, 1972). If this were the sole explanation for the shifts, then glycopeptide products, larger than the limit glycopeptides produced by Pronase digestion, should have been generated. This was not the case for any of the alkali-sensitive molecules examined. Consequently, it was concluded that the shifts observed after alkaline hydrolysis primarily reflected release of O-linked oligosaccharides.

Estrogen also caused these cells to express a smaller proportion of hydrophobic glycoconjugates in the cell-associated fraction. Morris et al. (1988) have reported similar effects of estrogen on expression of HS proteoglycans in this system. In this case, it appears that the action of the hormone is to stimulate metabolic turnover of the hydrophobic proteoglycans with a subsequent accumulation of the nonhydrophobic glycosaminoglycan chains. While this observation can account for the decrease in the hydrophobic, sulfated glycoconjugates, it is not clear if this is the explanation for the decreased expression of other hydrophobic glycoproteins. Since the pattern of glycoconjugates in the nonhydrophobic, cell-associated fraction so closely resembles that of the secreted fraction, it seems likely that many of these molecules are destined for secretion. In the case of HS proteoglycans, it has been shown that these molecules undergo both secretion and lysosomal degradation in these cells. In contrast, N-linked LAGs appear to be secreted without evidence of an intracellular degradative pathway (Dutt and Carson, unpublished results). It is possible that estrogen preferentially stimulates production of the nonhydrophobic glycoconjugates destined for secretion. Consistent with this hypothesis, we found that estrogen preferentially stimulated production of high molecular weight, secreted, N-linked glycoproteins.

Another intriguing possibility is that expression of the mature pattern of glycoprotein production by uterine epithelial cells requires development of the underlying stroma. Indeed, stromal cells have been shown to have profound influences on the behavior of epithelial cells in several cases (Cunha et al., 1983). It is clear that estrogen alone is unable to generate a mature phenotype in the uterine stroma (Fainstat, 1963; Martin & Finn, 1968). Consequently, estrogen-treated stromal cells of immature animals would not be expected to function as mature stroma. We are currently trying to develop appropriate coculture systems to determine if mature uterine stroma can restore a mature pattern of glycoprotein expression to immature uterine epithelia. Collectively, these approaches should allow us to determine the contributions of hormones as well as other uterine cell types to biochemical aspects of the development of mature, implantation-competent uterine epithelia.

ACKNOWLEDGMENTS

We thank A. Dutt, D. Farrar, M. C. Farach-Carson, S. R. Glasser, A. Jacobs, J. Julian, L. Rohde, and O. Wilson for their many helpful critiques and suggestions. We are indebted to Ellen Madson for her excellent typing of the manuscript.

Registry No. HS, 9050-30-0.

REFERENCES

Carson, D. D., Dutt, A., & Tang, J. P. (1987a) *Dev. Biol. 120*, 228–235.

Carson, D. D., Tang, J. P., & Hu, G. (1987b) *Biochemistry 26*, 1598–1606.

Carson, D. D., Tang, J. P., Julian, J., & Glasser, S. R. (1988) *J. Cell. Biol. 107*, 2425–2435.

Cunha, G. R., Shannon, J. M., Taguchi, O., Fujii, H., & Meloy, B. A. (1983) in *Epithelial mesenchymal interactions in development* (Sawyer, R. H., & Fallon, J. F., Eds.) pp 51–74, Praeger Press, New York.

Dutt, A., Tang, J. P., Welply, J. K., & Carson, D. D. (1986) *Endocrinology 118*, 661–673.

Dutt, A., Tang, J. P., & Carson, D. D. (1987) *Dev. Biol. 119*, 27–37.

Dutt, A., Tang, J. P., & Carson, D. D. (1988) *J. Biol. Chem. 263*, 2270–2279.

Fainstat, T. (1963) *Am. J. Anat. 112*, 337–370.

Farach, M. C., Tang, J. P., Decker, G. L., & Carson, D. D. (1987) *Dev. Biol. 723*, 401–410.

Fukuda, M. (1985) *Biochim. Biophys. Acta 780*, 119–150.

Glasser, S. (1985) in *Reproductive Toxicology* (Dixon, R. L., Ed.) pp 219–238, Raven Press, New York.

Irimura, T., & Nicolson, G. L. (1983) *Carbohydr. Res. 120*, 187–195.

Lee, M. C., Wei, T. C., Wan, Y. J., & Damjanov, I. (1983) *Histochemistry 79*, 365–375.

Maier, D. B., Newbold, R. R., & MacLachlan, J. A. (1985) *Endocrinology 116*, 1878–1886.

Martin, L., & Finn, C. A. (1968) *J. Endocrinol. 41*, 363–371.

Morris, J. E., Potter, S. W., & Gaza Belseco, G. (1988) *J. Biol. Chem. 263*, 4712–4718.

Plantner, J. J., & Carlson, D. M. (1972) *Methods Enzymol. 27*, 46–48.

Psychoyos, A. (1973) *Vitam. Horm. (N.Y.) 31*, 201–256.

Quarmby, V. E., & Korach, K. S. (1984) *Endocrinology 114*, 694–699.

Tang, J. P., Julian, J., Glasser, S., & Carson, D. D. (1987) *J. Biol. Chem. 262*, 12832–12842.

Teng, C. T., Walker, M. P., Bhattacharya, S. N., Klapper, D. G., DiAugustine, R. P., & McLachlan, J. A. (1986) *Biochem. J. 240*, 413–422.