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THE EFFECTS OF SEQUENTIAL ADMINISTRATION OF 17β-ESTRADIOL ON THE SYNTHESIS AND SECRETION OF SPECIFIC PROTEINS IN THE IMMATURE RAT UTERUS

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Summary—We report here results of a study of the effect of sequential administration of 1 μg 17β-estradiol *in vivo* on the incorporation of L-[^{35}S]methionine into specific proteins *in vitro* in the immature rat uterus. One-dimensional SDS–PAGE analysis of labeled secreted uterine proteins and cellular proteins extracted from the luminal epithelial and from the stroma plus myometrial uterine fractions revealed that estradiol preferentially stimulated the synthesis of 110 K, 74 K and 66 K secreted proteins, 180 K and 110 K epithelial proteins and a 175 K stroma-myometrial protein among others, while it decreased the relative rate of synthesis of a 32.5 K secreted protein and a 70 K stroma-myometrial protein. The 110 K protein, a secreted luminal epithelial protein whose labeling *in vitro* dramatically increased >60-fold per mg endometrial DNA after *in vivo* estrogen stimulation, may be a useful marker for studying estrogen action in the luminal epithelium of the immature rat uterus. Comparison of the secreted proteins labeled at 28 h (4 h after a second injection) and at 54 h (6 h after a third injection) revealed that estradiol effected a sequential change in the pattern of synthesis of secreted uterine proteins *in vitro*. Comparison of the number and magnitude of changes in the synthesis of specific proteins in the luminal epithelium and the stroma plus myometrium revealed that protein synthesis in the luminal epithelium is clearly more responsive to estradiol and readily distinguishable from the responsiveness of the stroma plus myometrium.

INTRODUCTION

For many years the rodent uterus has been used as the model system for the study of estrogen action. Stimulation of the rodent uterus by estrogenic hormones initiates a cascade of biochemical events that result in macromolecular synthesis and uterine growth [1, 2]. The assumption has been that at least some of these uterine responses are mediated through the interaction of estrogen with the cytosol-nuclear estrogen receptor system in the target uterine cell [1–3]. To understand the macromolecular details of how the estrogen–nuclear receptor complex interacts with the genome of the uterus to effect new RNA and protein synthesis, we need a good molecular marker for estrogen action, either a RNA or protein species. Considerable progress has been made in understanding the receptor mediated action of hormones in the chick oviduct, for example, where a few highly specific, hormone-responsive proteins constitute a major proportion of the total proteins synthesized after hormone treatment [4–6]. Although it has long been known that estrogens stimulate a general increase in protein synthesis in the rodent uterus [7, 8], no specific protein has been identified in this organ that has as substantial a response to hormone stimulation as the egg white proteins ovalbumin and conalbumin do in the chick oviduct.

Several uterine proteins have been suggested as potential markers of estrogen action. Induced protein (IP), first described by Notides and Gorski [9], is one such protein; its synthesis is one of the earliest responses to estrogen *in vivo* [10] and *in vitro* [11]. IP has recently been identified as not one but at least three proteins [12], the major component being the brain type (BB) isozyme of creatine kinase (CKBB) [13]. Estrogen-induced increases in the rate of CKBB synthesis have been reported in not only the uterus but also the rat pituitary, hypothalamus, brain cortex, liver [14, 15], the reticulo-epithelial cells of the thymus [16], the ovary [17], vagina [18], and may well be in the entire female reproductive tract of the immature rat [17, 19]. Other uterine proteins studied

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Abbreviations: *BIS*, *N,N'*-methylene-*bis*-acrylamide; BSA, Bovine serum albumin; DATD, *N,N'*-diallyltartardiamide; DTT, DL-dithiothreitol; E_2, 17β-estradiol, 1,3,5(10)-estratriene-3,17β-diol; MEM, Minimum essential medium; M_r, Molecular weight; PAGE, Polyacrylamide gel electrophoresis; SDS, Sodium dodecyl sulfate; TBSM, Tris-buffered saline containing unlabeled L-methionine; TCA, Trichloroacetic acid.

as potential markers of estrogen action are the estrogen receptor [20], progesterone receptor [21], hydrolyase [22–24], glucose-6-phosphate dehydrogenase [25], ornithine decarboxylase [26], peroxidase [27, 28], and recently plasminogen activator [29, 30]. Studies of the latter proteins have been based on biochemical assays of their enzymatic activity rather than determination of *de novo* protein synthesis. While enzymatic activity may correlate with *de novo* synthesis, there is accumulating evidence that the increased activity of certain enzymes in the uterus is not the result of *de novo* protein synthesis. For example, the major increase in peroxidase activity in the uterus following estrogen stimulation is due primarily to uterine eosinophilia in the stimulated uterus and the consequent increase in eosinophilic peroxidase [31, 32]. In this case increased enzymatic activity is the result of a non-genomic estrogen-induced response in the uterus that is independent of genomic activation [33–35].

To study the effect of estrogen on the *de novo* synthesis of specific proteins a number of investigators have analyzed protein synthesis in a homogenate of the whole uterus by either double-isotope labeling [36–41], one-dimensional [36–42], two-dimensional [43–44], or three-dimensional electrophoresis [12]. However, many were unable to detect significant changes in the synthesis of specific proteins other than IP. One explanation is that only a single time point was studied, and usually it was an early time point. A more probable explanation is that by using a homogenate of the whole uterus the response of a very heterogeneous population of cells was studied. The response of the whole uterus to estrogen represents the average of the responses of the individual cell types that comprise the uterus. The uterus is a complex organ composed of three different cell types: epithelium, stroma and myometrium. Each of the cell types respond to estrogen stimulation in a unique manner [45–47]. Histological studies have indicated that in the ovariectomized mouse uterus estrogen-induced proliferation is restricted to the epithelia, with the luminal epithelium undergoing marked hyperplasia [48]. Yet after estrogenic stimulation the luminal epithelium comprises only 10–12% of the uterine tissue volume and 20% of the total uterine cell population [49]. Therefore, in analyzing homogenates of the whole uterus changes in protein synthesis in the epithelial cells could be masked.

It is important to focus on the response of the individual target cell rather than on the response of the whole target organ. Smith *et al.*[50] used this approach and demonstrated an overall increase in protein synthesis in both stromal and epithelial cell fractions after estrogen stimulation and observed that the effects of estrogen were most marked in the epithelium. These early studies, however, failed to show changes in synthesis of specific proteins. Others have approached the study by analyzing estrogen-stimulated synthesis of specific proteins in primary cultures of the separated uterine cell types [44, 51]. However, it is difficult to maintain a pure population of each of the individual uterine cell types in culture and even more difficult to maintain a level of estrogen receptor in the cultured cell that is necessary for estrogen-responsiveness [52–54]. In such cultures the integrity of the stroma-epithelial interactions are destroyed. There is evidence from other hormone-responsive systems that there are stromal-derived factors that influence the hormone responsiveness and development of adjacent epithelium [55].

Cognizant of the limitations of these other approaches, several investigators analyzed protein synthesis in rodent uteri that were stimulated *in vivo* and labeled *in vitro* prior to separation into the stromal, myometrial and epithelial fractions [56–60]. Quarmby and Korach[60] recently described estrogen responsive protein synthesis in the individual uterine tissue compartments after 2 h of hormone treatment. Using a similar approach we have been studying the changes that occur in estrogen-responsive synthesis of specific rat uterine proteins during 3 days of estrogen treatment. We exposed the uterus *in vivo* to 17β-estradiol and analyzed the uterine proteins synthesized *in vitro*, not at a single time point but at various times during treatment. Our working hypothesis is that the organ *in vitro* reflects the *in vivo* hormonal and metabolic state of the uterus at the time of its excision. In this paper we report our initial findings. We observed that estrogen stimulates dramatic changes in the synthesis of specific secreted uterine proteins, changes that do not occur simultaneously but sequentially. These estrogen-responsive secreted uterine proteins are but a subset of the luminal epithelial proteins whose synthesis is markedly increased following estrogen stimulation. We can clearly distinguish these changes in endometrial protein synthesis from the relatively minor changes in protein synthesis in the stroma plus myometrical portion of the uterus. We can identify estrogen-regulated protein synthesis that is unique to each of the uterine fractions.

EXPERIMENTAL

Chemicals and buffers

17β-Estradiol (E_2), L-methionine, calf thymus DNA, bovine serum albumin (BSA), DL-dithiothreitol, and Tris were purchased from Sigma Chemical Co. (St Louis, MO). A stock solution of E_2 at 5 mg per ml absolute ethanol was diluted with 0.9% (w/v) NaCl to a final concentration of 10 μg steroid per ml 10% (v/v) ethanol for injections. The culture medium (Methionine-free MEM) was Eagle's (Modified) minimum essential medium with Earle's salts without methionine and glutamine (Flow Laboratories, McLean, VA) supplemented with 2 mM L-glutamine (Flow Laboratories) just prior to use, gassed with 5% CO_2–95% air, and maintained in a tightly closed flask during culture. Tris-buffered

saline containing carrier methionine (TBSM) consisted of 150 mM NaCl and 5 mM L-methionine in 10 mM Tris pH 7.4. NCS tissue solubilizer and L-[^{35}S]methionine (sp. act. $\geq$1000 Ci/mmol) were obtained from Amersham Corp. (Arlington Heights, IL). Toluene scintillator solution contained 7.45 g 2,5-diphenyloxazole (PPO; RPI, Research Products International Corp., Elk Grove Village, IL) and 0.3 g 1,4-*bis*[2-(4-methyl-5-phenyloxazolyl)]benzene (dimethyl-POPOP; RPI) per L toluene. All chemicals used for SDS–PAGE were electrophoresis purity grade and were obtained from either Bio-Rad Laboratories (Richmond, CA) or Bethesda Research Laboratories, Inc. (Gaithersburg, MD) except for DATD, which was purchased from Aldrich Chemical Co. (Milwaukee, WI). Glutaraldehyde (50% in water) was obtained from Eastman Kodak Co. (Rochester, NY) and silver nitrate (silver stain grade) from Accurate Chemical and Scientific Corp. (Westbury, NY).

Treatment of animals

Immature (22-day old) female Sprague–Dawley derived rats (King Animal Labs, Oregon, WI), initially weighing 35–45 g, were injected s.c. once daily for 3 days with either 1 μg E_2 in 0.1 ml 10% ethanol in saline or 0.1 ml 10% ethanol in saline alone. The injections were at precisely 0, 24 and 48 h. At 28 h (4 h after the second injection) and 54 h (6 h after the third injection) 10 experimental animals and 25 control animals were sacrificed by decapitation; their uteri were quickly removed, dissected free of fat and connective tissue, weighed and separated into individual horns.

Incorporation of L-[^{35}S]*methionine by uterine tissue* in vitro

The uterine horns were slit open longitudinally and immediately incubated at 37°C in a Dubnoff Metabolic Shaking Incubator, first for 1 h in methionine-free MEM and then for 3 h in fresh medium containing 100 μCi L-[^{35}S]methionine (sp. act. $\geq$1000 Ci/mmol) per ml methionine-free MEM. For labeling the tissue was incubated at 75 mg (tissue wet wt) per ml medium. The 1 h preincubation in the absence of methionine, together with labeling in methionine-deficient medium, increased the specific activity of total uterine protein in estrogen-treated tissue. We found that without preincubation in the absence of methionine the specific activity of total uterine cellular protein decreased as a function of time after estrogen administration (unpublished data). It is probable that this decrease is due to an increase in the size of the intracellular amino acid pool effected by estradiol in the rat uterus [61]. To monitor the *in vitro* incorporation of L-[^{35}S]methionine into uterine protein, duplicate tissue samples were removed after each hour of incubation, rinsed in excess ice-cold TBSM, and homogenized in 1 ml 10 mM Tris pH 7.4 containing 5 mM carrier methionine. An aliquot of the homogenate was precipitated with 1 ml 10% TCA in the presence of 5 mM methionine and heated at 90°C for 30 min. The precipitate was collected on Whatman GF/C glass fiber filters, washed successively with first 10% TCA containing 5 mM carrier methionine, then 2% potassium acetate in 75% ethanol, and finally 75% ethanol, and partially air dried. To solubilize the precipitate 0.5 ml NCS:H_2O (9 vol NCS to 1 vol H_2O) was added to the filter and heated at 50°C for 30 min, after which the solution was neutralized with glacial acetic acid and counted in 10 ml toluene scintillator solution in a Tri-Carb 300C liquid scintillation counter (Packard Instruments Co., Inc., Downers Grove, IL). ^{35}S was counted at 93% efficiency. Under these conditions the incorporation of L-[^{35}S]methionine into total cellular protein was linear for the 3 h incubation period, and total uterine protein was labeled to a specific activity of $\geq 1 \times 10^7$ dpm/mg protein by 3 h. To terminate the incorporation of labeled methionine the tissue was removed from culture and rinsed thrice in excess ice-cold TBSM. The incubation medium was cooled on ice, clarified of cellular contaminants by centrifugation at 500 ***g*** for 10 min at 4°C, dialyzed exhaustively at 4°C against TBS, and concentrated to 1 ml using a multiple dialysis-concentrator (Micro-ProDiCon, Bio-Molecular Dynamics, Beaverton, OR). The protein concentration of the concentrate was measured by the Coomassie brilliant blue dye-binding method of Bradford[62], as modified by Bearden[63], using BSA as the standard. To determine the specific activity of the media proteins, duplicate assays of the concentrate were precipitated with 1 ml 10% TCA in the presence of 125 μg carrier BSA and heated at 90°C for 30 min. The TCA precipitable radioactivity was collected on filters and counted as described above.

Separation of luminal epithelium

After incubation the uterine tissue was physically separated into one fraction highly enriched for the luminal epithelium and a second fraction consisting of the residual stroma plus myometrium, using the non-enzymatic method of Fagg *et al.*[64]. Briefly, 2–4 slit uterine horns (75–100 mg total tissue wet wt) were vortexed at maximum speed for 2 min in 1 ml ice-cold TBSM in a 15 ml round-bottom test tube with eight 4 mm dia glass balls. All procedures were performed at 4°C unless stated otherwise. The "residual" uterine horns were removed from the suspension of epithelial cells, rinsed in TBSM, a small portion fixed for histology, and the remainder pooled and homogenized in 4 vol of 10 mM Tris pH 7.4, using a Polytron PT-10 homogenizer (Brinkmann Instruments Inc., Westbury, NY). As we previously observed [31], histological examination revealed that the "residual" horn consisted of the myometrium, endometrial stroma, and some of the deeper glandular epithelium. Left suspended in the saline was virtually all of the luminal epithelium and part of the glandular epi-

thelium. The "epithelial fraction" was collected by centrifugation of the cell suspension at 1000 *g* for 15 min and homogenized in 1 ml 10 mM Tris pH 7.4. Duplicate assays of the homogenates of both the "epithelial" and "residual" stroma plus myometrial fractions were taken for DNA determination as measured by the method of Burton[65] using calf thymus DNA as standard. The homogenates were centrifuged at 27,000 *g* for 45 min. The supernatants (designated 10 mM Tris extracts) were prepared for SDS–PAGE analysis as described below, while the pellets were rehomogenized in the same volume of 1 N NaCl in 10 mM Tris pH 7.4. After a 30 min incubation at 4°C, the homogenates were centrifuged at 27,000 *g* for 45 min and the supernatants (designated 1 N NaCl Extracts) were prepared for SDS–PAGE analysis. The total protein and radioactivity in each extract were determined as described above and expressed relative to the DNA content of the respective uterine fraction.

SDS-polyacrylamide gel electrophoresis

One-dimensional denaturing polyacrylamide gel electrophoresis was done using the discontinuous buffer system of Laemmli[66] with the following modifications: The separating gel (14 cm × 28 cm × 0.75 mm) consisted of 15% (w/v) acrylamide, 0.39% (w/v) DATD, 0.375 M Tris pH 8.8, and 0.1% SDS and the stacking gel, 1 cm in length, consisted of 7% acrylamide, 0.19% *BIS*, 0.125 M Tris pH 6.8, and 0.1% SDS. The electrode buffer contained 0.025 M Tris pH 8.3, 0.192 M glycine, and 0.1% SDS. DATD [67] was used as the cross-linker in the resolving gel primarily to improve the pliability and drying of the gel. The protein in the tissue extracts and concentrates of the media was precipitated with ice-cold 10% TCA, pelleted, washed and then resuspended in SDS–PAGE sample buffer (50 mM Tris pH 6.8, 2% SDS, 5% β-mercaptoethanol, 10% sucrose and 0.005% bromophenol blue) at approx 3 mg per ml. If necessary, the resuspension was adjusted to neutral pH with 2 M Tris pH 8.8 and ultrasonicated twice for 10 s at maximum output with intermittent cooling. To denature and solubilize the protein each sample was heated at 80°C for 4 min. Equal volumes (25 μl) of the samples containing either equivalent amounts of radioactivity (50,000–200,000 dpm) or equivalent amounts of protein (5 μg) were layered onto the gel. Electrophoresis was carried out at 4°C in a temperature-controlled buffer chamber (Hoefer Scientific Instruments, San Francisco, CA) at a constant current of 7.5 mA per gel until the bromophenol blue dye marker had migrated 13 cm into the resolving gel (about 14 h). The following protein molecular weight standards were electrophoresed under identical conditions and the apparent M_r of a protein was determined relative to their migration: myosin 200,000 M_r β-galactosidase 116,500 M_r, phosphorylase B 94,000 M_r, BSA 68,000 M_r, ovalbumin 43,000 M_r, carbonic anhydrase 30,000 M_r, soybean trypsin inhibitor 21,000 M_r and lysozyme 14,300 M_r (Bio-Rad Laboratories, Richmond, CA).

Immediately following electrophoresis the gel was fixed in 20% isopropanol–10% glacial acetic acid for 1 h, washed for 1 h in running distilled water, and then stained with silver according to the technique of Switzer *et al.*[68], as modified by Oakley *et al.*[69], Morrissey[70], and Wray *et al.*[71]. Briefly, to maximize the sensitivity of the silver stain the washed gel was fixed for 30 min in freshly prepared 10% unbuffered glutaraldehyde, after which it was rinsed extensively in running distilled water. To ensure uniform and reproducible staining the gel was soaked for 30 min in aq. DTT at 5 μg/ml and then rinsed in running distilled water for 30 min. The gel was stained for 15 min in a freshly made ammoniacal silver nitrate solution containing 21 ml 0.36% NaOH, 1.4 ml fresh 14.8 M NH_4OH, 74 ml 20% ethanol and 4 ml 20% aq. $AgNO_3$. After staining the gel was washed in running distilled water for 20 min and then developed in a freshly prepared reducing solution of 0.005% citric acid, 0.0185% formaldehyde and 10% ethanol until the desired intensity of staining was attained. Staining was stopped by quickly washing the gel in several changes of distilled water. Densities obtained with this silver staining procedure were proportional over a wide range of protein concentrations. After a final exhaustive washing in running distilled water, the stained gel was dried under vacuum with heat onto a single sheet of porous cellophane on a slab gel drier (Hoefer Scientific Instruments).

For autoradiography the dried gel was exposed directly to Dupont Cronex 4 medical X-ray film (E. I. du Pont de Nemours and Co., Inc., Wilmington, DE) at room temperature for 3–7 days. Film was developed at 20°C for 3 min in Kodak GBX developer, rinsed for 20 s in water, fixed for 6 min in Kodak rapid fixer with hardener, and washed for 20 min in running water before a final rinse for 30 s in Kodak Photo-Flo 200 solution. In preliminary studies we demonstrated that under these conditions there is a linear relationship between subsaturating image density and increasing ^{35}S concentration over the range of measurable dpm applied to the gels.

Densitometric scanning of stained gels and autoradiograms

The optical density of the silver stained gels and corresponding autoradiograms was scanned at a resolution of 50 μm with a soft laser scanning densitometer (manufactured by Biomed Instruments, Inc., Chicago, IL, for LKB Instruments, Inc., Gaithersburg, MD). With the aid of a Digital PDP-11/24 computer and graphics display terminal (Tektronix 4010-1; Tektronix, Inc., Beaverton, OR) data analysis was carried out on specific, discrete polypeptide bands (1) to determine the apparent M_r of proteins based on their migration relative to that of protein

molecular weight standards whose subunit molecular weights are known, (2) to integrate the density of specific bands, and (3) to align and plot several scans simultaneously on a Zeta 8 digital plotter (Nicolet Zeta Corp., Concord, CA). The relative amount of L-[^{35}S]methionine incorporated into a specific protein was approximated by integration of its peak in the densitogram of the autoradiogram. The relative rate of incorporation (R) of L-[^{35}S]methionine into a specific protein (i) at a specified time (t) is expressed using the following relation:

$$R_i = K_t \cdot \frac{A_i}{\Sigma A_i \ldots A_n}$$

where K_t is the overall rate of incorporation of L-[^{35}S]methionine into total protein at the time of determination, expressed as dpm per mg DNA, A_i is the density specifically associated with polypeptide band i, and $\Sigma A_i \ldots A_n$ is the total density of the scan.

RESULTS

Secretory uterine proteins

Immature female rats were administered daily s.c. injections of 1 μg 17β-estradiol for three consecutive days. At specific times during treatment the animals were sacrificed, the uteri removed, and the whole

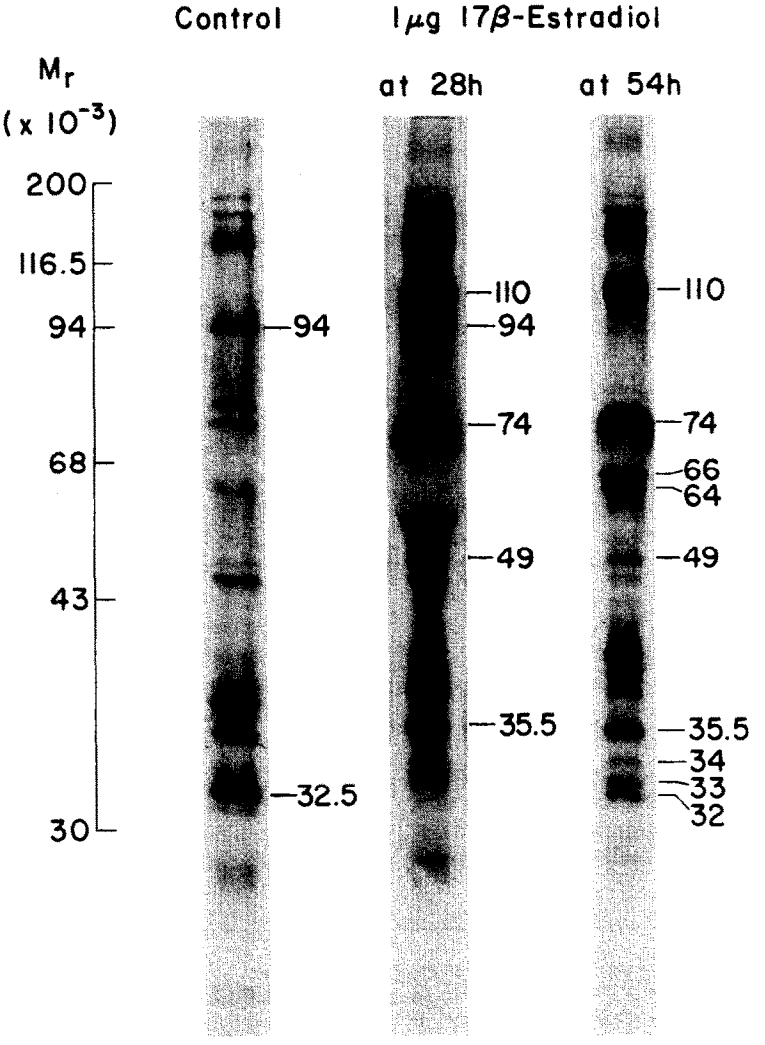


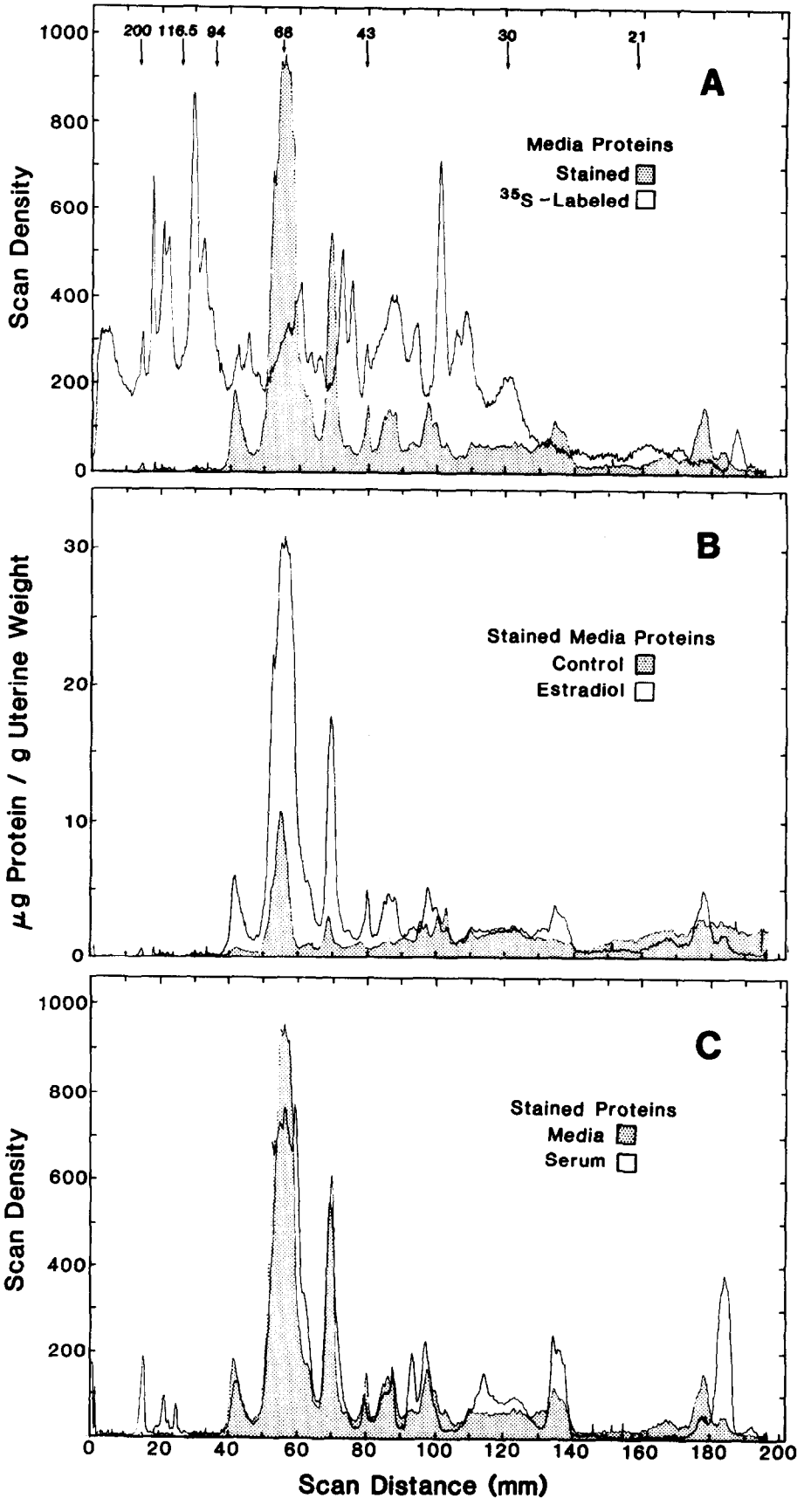
Fig. 1. Autoradiographic image of the SDS–PAGE analysis of uterine proteins released *in vitro* at 28 and 54 h. Twenty-two-day old rats were injected s.c. with either saline (Control) or 1 μg 17β-estradiol at 0, 24 and 48 h. Uteri were removed at 28 h (4 h after the second injection) and 54 h (6 h after the third injection) and incubated *in vitro* for 3 h with 100 μCi L-[^{35}S]methionine per ml medium. The protein in the medium was precipitated with 10% TCA and 50,000 dpm of each sample was analyzed on one-dimensional SDS–PAGE as described in the Experimental section. The proteins are designated by their relative molecular weight (M_r) which is determined relative to the migration of the following protein molecular weight standards analyzed on the same gel: myosin (200,000 M_r), β-galactosidase (116,500 M_r), phosphorylase B (94,000 M_r), bovine serum albumin (68,000 M_r), ovalbumin (43,000 M_r) and carbonic anhydrase (30,000 M_r). The distortion in the gel in the 68,000 mol. wt range (see 28 h E_2 sample) is due to the high concentration in the media of a complex of proteins, the majority of which do not incorporate L-[^{35}S]methionine.

uterus cultured *in vitro* for 3 h with high specific activity L-[^{35}S]methionine. In the first series of experiments we analyzed the labeled proteins released into the media during the *in vitro* incubations. We observed a greater than 5-fold increase in the overall rate of incorporation of L-[^{35}S]methionine into secreted uterine proteins *in vitro* following *in vivo* estradiol treatment (average of five separate experiments). To determine whether this increased synthesis included a qualitative change in the pattern of synthesis of secreted uterine proteins, we compared the labeled proteins released into the medium by uteri of control and E_2-treated animals at 28 h (4 h after the second injection) and 54 h (6 h after the third injection). Figure 1 shows an autoradiographic image of a typical one-dimensional SDS–PAGE analysis of these proteins. Here equivalent amounts of radioactivity of each sample are compared to enhance the qualitative differences in labeling between samples. It is obvious from the protein profiles that at 54 h there are dramatic differences in the synthesis of secreted proteins in control and E_2-treated uterine tissue. There are in the E_2 sample labeled 110K, 74K, 66K, 34K, 33K and 32K proteins that are not apparent in the comparable control sample. Two of these proteins, the 110K and 74K secreted proteins, account for approx 10 and 15%, respectively, of the total labeling of media proteins in E_2-treated tissue at 54 h. Quantitation of the relative rate of incorporation of L-[^{35}S]methionine into specific proteins (expressed as dpm per mg endometrial DNA) revealed that in E_2-treated tissue these two proteins each showed a greater than 60-fold increase in the rate of labeling by 54 h compared to controls, increases that are dramatically greater than the 5-fold increase in overall labeling of media proteins. We also observed that *in vivo* estradiol administration increased the relative rate of synthesis of the 49K and 35.5K proteins but decreased the relative rate of labeling of the 94K and 32.5K proteins at 54 h. These effects on the synthesis of specific secreted proteins are estrogen-specific in that hydrocortisone, dexamethasone, progesterone, the synthetic progestins megestrol acetate and medroxyprogesterone acetate, 5α-dehydrotestosterone, and physiological concentrations of testosterone do not elicit these responses. Other estrogens do elicit these responses and at a magnitude that reflects their differential estrogenic potency (diethylstilbestrol > 17β-estradiol > estrone > estriol > 17α-estradiol) [manuscript in preparation].

Comparison of the profiles of media proteins synthesized by E_2-treated tissue at 28 and 54 h reveals that not all of the changes evident at 54 h are evident at 28 h. At 28 h there is synthesis of the 110K and 74K proteins in E_2-treated tissue but the relative rate of labeling of the 110K protein, for example, is increased only 10-fold compared to controls. At 28 h the relative rate of synthesis of the 49K and 35.5K proteins is increased. Although the relative rate of synthesis of the 32.5K protein is decreased by 28 h, the synthesis of the 94K protein is not. This comparison suggests that the observed changes in synthesis of media proteins do not occur simultaneously and that the time course of response of specific proteins to *in vivo* estrogen stimulation may be different. A detailed time study of synthesis of individual secreted proteins at specific times throughout hormonal treatment confirmed this initial observation and revealed that *in vivo* estradiol treatment effects a sequential change in the synthesis and secretion of specific uterine proteins *in vitro* (manuscript in preparation).

We have assumed thus far in this study that an increase in a labeled secreted protein results from an increase in its synthesis; however, it is equally conceivable that it could result from increased secretion of its labeled cellular precursor. To differentiate between these possibilities we need to establish the effect of *in vivo* estradiol treatment on the synthesis of cellular uterine proteins and its effect on the release of protein by uterine tissue *in vitro*. A comparison of the media proteins that incorporate L-[^{35}S]methionine *in vitro* and those that stain with silver (Fig. 2A) reveals that the media proteins that are labeled *in vitro* do not correspond to the major silver staining components of the media and constitute only a minor proportion of the proteins that accumulate in the media during the 3 h *in vitro*

Fig. 2. Comparison of the proteins that are released into the medium by E_2-treated uterine tissue at 28 h and that stain with silver and (A) those media proteins that are labeled with L-[^{35}S]methionine at 28 h, (B) the proteins that are released into the medium by control uterine tissue at 28 h and that stain with silver, and (C) serum proteins from a 23-day old, E_2-treated rat. A. A plot of a densitometric scan of the silver stained image (shaded plot) of the SDS–PAGE analysis of the uterine proteins released *in vitro* by E_2-treated tissue at 28 h superimposed on a plot of a scan of its autoradiographic image (unshaded plot). Scan density is plotted vs scan distance (mm). The arrows designate the relative migration distances of the protein molecular weight standards. B. Superimposed plots of the densitometric scans of the silver stained images of the SDS–PAGE analysis of proteins released *in vitro* by control uterine tissue (shaded) and E_2-treated uterine tissue at 28 h (unshaded). The total protein that accumulated in the medium during each incubation was calculated, TCA precipitated, and 5 μg of each was analyzed by SDS–PAGE as described in the Experimental section. The densitometric scans are plotted vs the overall rate of secretion of uterine proteins at 28 h, which is expressed as μg media protein per g uterine wet weight incubated *in vitro* for 3 h. C. Superimposed plots of the densitometric scans of the silver stained images of the SDS–PAGE analysis of proteins released *in vitro* by E_2-treated uterine tissue at 28 h (shaded plot) and serum proteins from the same animals (unshaded plot).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incubation. To determine whether *in vivo* estradiol treatment alters the secretion of uterine proteins *in vitro* we compared the stained proteins that accumulate in the media during the 3 h *in vitro* incubation of control and E_2-treated uterine tissue and observed that *in vivo* estradiol treatment stimulates at least a 2-fold increase in the overall rate of release of protein *in vitro* at the 28 h time point. To determine whether the secretion of all proteins is uniformly stimulated we compared the specific proteins released by control and E_2-treated tissue and observed a preferential increase in the rate of release of the 86K, 52K, 43K and 36K proteins as well as a complex of proteins in the 68K and 39–42K molecular weight range (Fig. 2B). We have also observed a decrease in the relative rate of release of a 90K protein after estrogen treatment (data not shown). These are media proteins that we showed in Fig. 2A do not incorporate L-[^{35}S]methionine *in vitro*, suggesting that their increased release by E_2-treated uterine tissue is not the result of new synthesis. Comparison of the electrophoretic separation of these media proteins with serum from the same animals (Fig. 2C) revealed that several of these media proteins (86K, 68K, 52K, 43K, 36K proteins and the proteins in 39–42K molecular weight range) co-electrophorise with serum proteins. Their identification as specific serum proteins has yet to be confirmed by other methods. Comparison of the scans plotted in Fig. 2C reveals, however, that there are several serum proteins (200K, 149K and 38K proteins) that are not present in the same relative concentrations in the media and serum. The data suggests that there is differential uptake and/or release of serum proteins by uterine tissue following *in vivo* estrogen treatment.

Intracellular uterine proteins

In the next series of experiments we compared the synthesis of specific proteins in the luminal epithelium and the stroma plus myometrium of the immature rat uterus. Our objectives were 3-fold: to determine if we could detect cellular proteins in the immature rat uterus whose synthesis is responsive to estradiol, to determine if from analysis of the synthesis of specific proteins we could distinguish the responsiveness of the uterine luminal epithelium from the responsiveness of the residual stroma plus myometrium, and finally to determine the cellular origin of those secretory proteins whose synthesis is responsive to estradiol. To study the proteins in the individual uterine fractions that incorporated L-[^{35}S]methionine *in vitro*, we physically separated the luminal epithelium from the residual stroma plus myometrial portion of the uterus after the labeling step. SDS–PAGE analysis of the labeled proteins in each fraction readily revealed that the cellular protein profile of either fraction was more complex than the profile of media proteins. To reduce the complexity of the protein population we sequentially extracted each fraction first with 10 mM Tris pH 7.4 and then with 1 N NaCl in Tris buffer prior to SDS–PAGE analysis.

Analysis of the proteins in the 10 mM Tris extract of the luminal epithelium revealed a 5-fold increase in the overall rate of synthesis of these proteins in E_2-treated tissue at 54 h when expressed as dpm per mg DNA (average of five separate experiments). To determine whether the synthesis of all proteins in this extract is uniformly increased, we compared the specific proteins synthesized in control and E_2-treated tissue at 54 h. In Fig. 3, which shows an autoradiographic image of a typical SDS–PAGE analysis of the proteins extracted from the respective fractions of control and E_2-treated tissue at 54 h of treatment, equivalent amounts of radioactivity are compared to show the qualitative differences in labeling between the two samples. It is obvious that the profiles of the labeled proteins in the 10 mM Tris extract of the luminal epithelium of control and E_2-treated tissue are different. In the E_2-treated tissue extract there are labeled 180K, 110K, a complex of proteins in the 68–75K molecular weight range, 61K, 53K, and 39K proteins that are not apparent in the comparable extract of control tissue. These proteins either are not synthesized in control tissue or are synthesized at much lower rates. Analysis of the proteins in the 1 N NaCl extract of the luminal epithelium revealed an 3-fold increase (average of five separate experiments) in the overall rate of synthesis of these proteins after 54 h of hormone treatment. Figure 3 reveals that there is also a dramatic difference in the labeling of specific proteins in this extract in E_2-treated and control tissue. In the 1 N NaCl extract of luminal epithelium from E_2-treated rats there are labeled 180K, a complex of labeled proteins in the 135–155K molecular weight range, 110K, 52K, 43K, 42K and 36K proteins that are not evident in the profile of labeled proteins in the comparable extract of control tissue. Whether the proteins of comparable molecular weight in the two extracts of the luminal epithelium of E_2-treated tissue are the same protein, different proteins, or different processed forms of the same protein reflecting differential extractability has yet to be determined. These results clearly show a major change in the pattern of protein synthesis in the rat luminal epithelium after estradiol treatment. It should be noted that based on DNA determinations this fraction of the uterus constitutes less than 10% of the total uterine cell population.

SDS–PAGE analysis of the labeled proteins in the 10 mM Tris and 1 N NaCl extracts of the stroma plus myometrium (Fig. 3) revealed that the stroma-myometrial proteins labeled *in vitro* at 54 h in E_2-treated and control tissue are qualitatively quite similar. Other than the dramatic increase in the synthesis of the 43K protein, we observed only a few changes in the pattern of protein synthesis in the stroma plus myometrium after estradiol treatment. *In vivo* estradiol administration consistently suppressed the relative rate of synthesis of the 90K and 76.5K

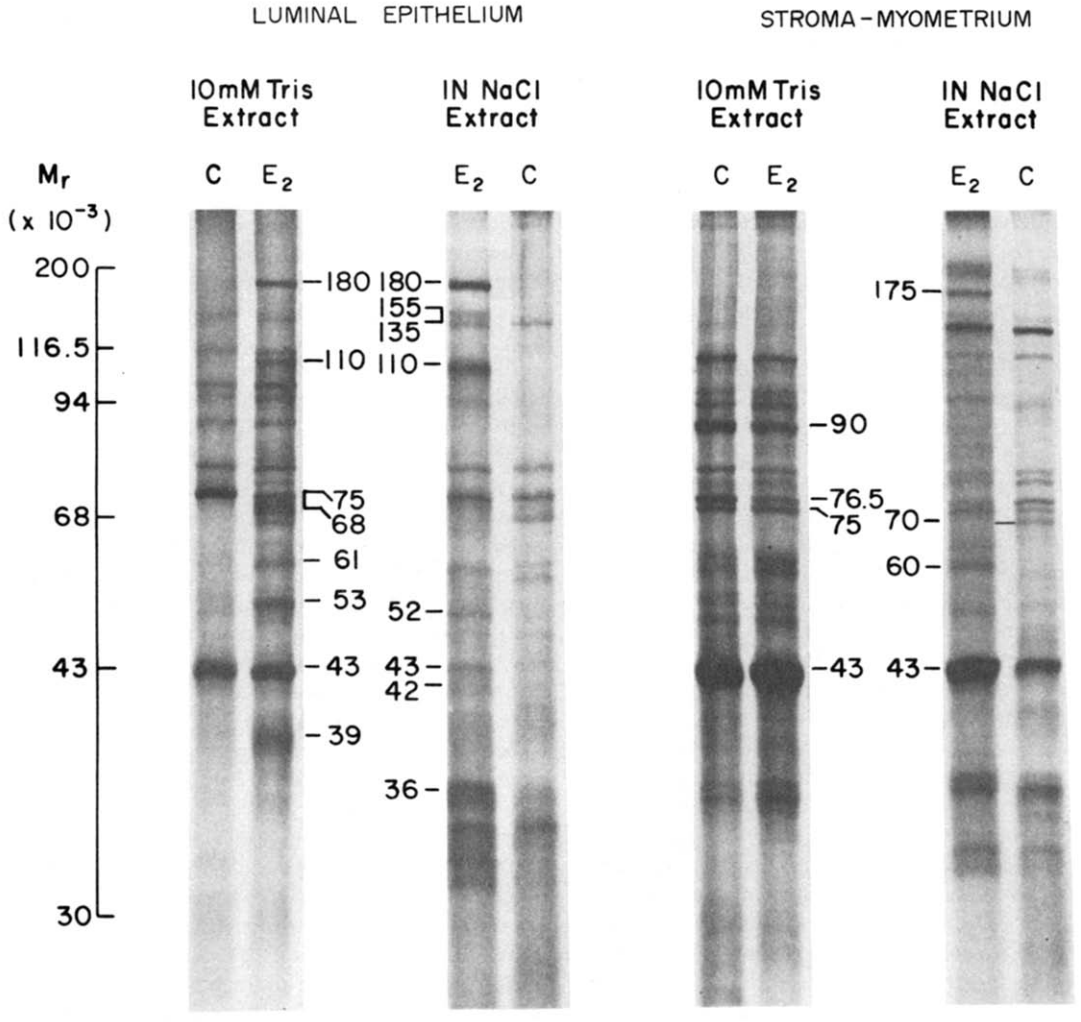

Fig. 3. Autoradiographic image of the SDS–PAGE analysis of the cellular uterine proteins synthesized *in vitro* at 54 h. Twenty-two-day old rats were injected s.c. with either saline (C) or 1 μg 17β-estradiol (E_2) at 0, 24 and 48 h. At 54 h (6 h after the third injection) the uteri were removed and incubated *in vitro* for 3 h with 100 μCi L-[^{35}S]methionine per ml medium, after which they were separated into the luminal epithelial and stroma plus myometrial uterine fractions. Each fraction was homogenized in 0.01 M Tris pH 7.4 and separated into the supernatant (10 mM Tris extract) and residual tissue pellet. The pellet was rehomogenized in 1 N NaCl in 0.01 M Tris pH 7.4 and centrifuged. The supernatant from this second centrifugation is the 1 N NaCl extract. The protein in each extract was precipitated with 10% TCA and equivalent amounts of radioactivity (approx 150,000 dpm) of each sample was analyzed on one-dimensional SDS–PAGE as described in the Experimental section. The proteins designated by their relative M_r are those whose rate of incorporation of L-[^{35}S]methionine *in vitro* in E_2-treated tissue changed relative to the control rate.

proteins, present in the 10 mM Tris extract of the stroma plus myometrium, and a 70K protein, characteristically extracted from this fraction with 1 N NaCl. There is in the 1 N NaCl extract of E_2-treated tissue at 54 h a labeled 175K protein that is not evident in the comparable extract of control tissue. This protein either is not synthesized in control tissue or is synthesized at enhanced rates only after estrogen stimulation. These changes in protein synthesis in the stroma plus myometrium, though fewer and less dramatic, are distinctly different from the changes in protein synthesis observed in the luminal epithelium. Progesterone does not elicit these changes in *in vitro* synthesis of intracellular proteins in the luminal epithelium or in the stroma plus myometrium of the immature rat uterus (manuscript in preparation).

DISCUSSION

The results presented in this study clearly show that there are dramatic changes in the labeling of tissue and secreted proteins of the immature rat uterus following estradiol treatment. The major L-[^{35}S]methionine-labeled secretory proteins, the 110K and 74K proteins, are probably products of the

luminal epithelial cells of the endometrium. A labeled 110K protein is found in both the 10 mM Tris and 1 N NaCl extracts of the epithelial cells, whereas no significant 110K protein is extracted from the stroma plus myometrium. By 54 h of estradiol treatment, the 110K protein can be detected as a distinct silver stained band on SDS–PAGE gels, constituting about 5% of the silver stained protein of the medium as well as a significant labeled component of the extracts of the luminal epithelium. It is a good candidate for a marker protein for studying estrogen action in the rat endometrium. Since there is a labeled 75K protein extracted from the stroma plus myometrium as well as from the epithelium, the origin of the other major estrogen-stimulated secretory protein is less certain. Further studies will be necessary to identify the exact nature and origin(s) of the 74K secreted protein.

It is clear that *in vivo* estradiol treatment has a most profound effect on the expression and secretion of proteins by the uterine epithelium *in vitro*. Major increases in synthesis of more than a dozen epithelial cellular and secreted proteins are seen after several days of estrogen treatment. It is also of interest that some of these changes in the labeling pattern of secreted proteins are clearly related to the time of estradiol treatment. For example, it is evident in Fig. 1 that there is a substantial increase in the proportion of labeling found in the 49K protein band at 28 h. The 94K secreted protein seen in the control is still a significantly labeled protein band at 28 h. However, at 54 h the labeled 49K protein appears to be a decreased proportion of the total labeled media proteins and the rate of labeling of the 94K protein is substantially suppressed. The labeling of the 110K and 74K proteins, which are both evident at the earlier time point, continues to increase throughout these times. The 35.5K protein also seems to show a continued increase with duration of estrogen treatment. On the other hand, the 66K and 32K, 33K and 34K proteins seem to be most evident at the later time point. Thus the 49K protein seems to be mainly an early responsive protein, while the 66K, 32K, 33K and 34K proteins are late responsive proteins. It is apparent that *in vivo* estrogen treatment effects a sequential change in uterine protein synthesis *in vitro*, involving increased synthesis of a number of new secreted epithelial proteins and decreased synthesis of other proteins in a distinct temporal pattern. Obviously a more detailed analysis in which more time points are studied during estradiol treatment will be needed to define in detail the temporal relationships of the estrogen-induced changes in secreted proteins of the uterus. Such studies are in progress.

It has been known for a long time that estrogen treatment causes a multitude of changes in the rat uterus, including changes in the quantity, activity and nature of proteins synthesized. However, only by looking at the individual cell types of the uterus can one begin to unravel the complex nature of the hormonal regulation of this reproductive organ. As pointed out earlier, hormones may have opposite effects on the different cell types of the uterus so that previous studies of hormone effects on even specific proteins or enzymes of the whole uterus can be misleading when used to understand the function of the organ.

When the labeled proteins from the luminal epithelium and from the stroma plus myometrium (shown in Fig. 3) are compared, it is evident why many of the earlier studies of differential labelling of proteins extracted from the whole uterus were not able to identify the very substantial differences in L-[^{35}S]methionine labeling reported here. While there are some cellular proteins of the stroma plus myometrium whose apparent synthetic rates are changed after estradiol treatment, most of the changes observed are found in the extracts of the luminal epithelium. These results are consistent with earlier work that demonstrated the differential responses of the uterine cell types to hormone treatment [45] and that showed the effects of estrogen stimulation are most marked in the luminal and glandular epithelia. The labeling of the 43K region in both the epithelium and residual uterus is one example in which estradiol seems to have a similar effect in both portions. This is the region in which the estrogen-regulated creatine kinase (approx 44,000 M_r) [12, 13], the major component of IP, is found and also actin (43,000 M_r) migrates. In the epithelium the significantly increased synthesis corresponding to this molecular weight range is seen largely in the low salt extract, as is consistent with the known extractability of the IPs. Fewer distinctive changes were seen in the stroma plus myometrium. Most dramatic is the substantial increase in the labeling of 43K proteins in the 10 mM Tris and 1 N NaCl extracts. A significant increase in the labeling of a 175K protein, characteristically extracted from these cells with 1 N NaCl, was evident, as was a decrease in the relative rate of synthesis of a 70K protein.

Recently Quarmby and Korach[60] reported two-dimensional electrophoretic analysis of mouse uterine proteins labeled with L-[^{35}S]methionine *in vitro*. They reported that estradiol has a significant effect on protein synthesis in the uterine epithelium, showing changes not evident when the whole uterus was analyzed. They also observed certain uterine proteins whose synthesis was depressed after estradiol treatment, indicative of the shift in protein synthesis described above. Since these authors studied an early time point (2 h after E_2 treatment) the identity of protein changes are different than those reported here. Nonetheless, despite differences in species and time of treatment, it is clear from the results of Quarmby and Korach[60] and those reported here that estradiol treatment influences the protein synthesis of each of the cell types of the rodent uterus in a distinctly different manner.

It is interesting that secretory proteins, such as the 110K uterine protein, whose incorporation of

L-[^{35}S]methionine is stimulated to such a significant degree have not been previously reported. One possible explanation is that it is clearly a late response to estrogen. In studies to be reported elsewhere, we found that the increased labeling of the 110K protein can be detected earlier than 28 h, but its presence as a distinct band in autoradiograms of media proteins is not obvious until 8–12 h of estrogen treatment. Therefore, because of the intense interest in the identification of early responsive proteins as key intermediary proteins in estrogen regulation of growth, late responsive proteins may have been missed. It is also possible that the 110K protein is rich in methionine but deficient in leucine, the labeled amino acid precursor used in many of the early experiments which looked at the differential labeling of rat uterine proteins during estrogen treatment.

It appears that the major silver staining proteins that accumulate in the *in vitro* incubation media correspond to serum proteins based on their relative migration on SDS–PAGE gels. Yet the profile of stained secreted uterine proteins is clearly different from the profile of rat serum proteins. Absent in the pattern of secreted uterine proteins are some of the larger serum proteins. There is no evidence that the major stained proteins are labeled with L-[^{35}S]methionine *in vitro*, consistent with their origin in serum and uptake into the uterus as a result of the changes in the permeability of the uterus following *in vivo* estrogen treatment. Furthermore, it is evident from Fig. 2 that *in vivo* hormone treatment clearly increases the secretion of uterine proteins *in vitro*. It would appear then that the uterus is selective in respect to the serum proteins that it secretes. Others have observed selective uptake and secretion of serum proteins into the luminal fluid in the estrogen stimulated uterus [72–75].

Finally, one of our goals in these studies was to be able to identify some major estrogen-responsive proteins that could serve as useful marker proteins to study the details of estrogen regulation of uterine growth and function. As demonstrated in this paper there are indeed a number of estrogen-responsive proteins whose rate of synthesis, as suggested by precursor incorporation *in vitro*, indicate major increases following treatment with the hormone *in vivo*. The more than 60-fold increases in labeling of the 110K and 74K secreted proteins make them good candidates for such markers. Current efforts in our laboratory are directed to the isolation and characterization of these major estrogen-responsive proteins of the rat endometrium. It is obvious that the nature and function of these estrogen-regulated proteins will be of significant value for understanding the nature of the endocrine control of the function of the endometrium.

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