Biochemical Characterization and Biosynthesis of the Uterine Milk Proteins of the Pregnant Sheep Uterus

PETER J. HANSEN,2,7 NANCY H. ING,8 R. JEFFREY MOFFATT,3,9 GEORGE A. BAUMBACH,4,8 PHILIPPA T.K. SAUNDERS,5,8 FULLER W. BAZER9 and R. MICHAEL ROBERTS6,8

Departments of Reproduction,7 Biochemistry and Molecular Biology8 and Animal Science9 University of Florida Gainesville, Florida 32610

ABSTRACT

The uterine milk proteins (UTM-proteins), a pair of basic glycoproteins with similar isoelectric points and molecular weights (57,000 and 55,000), are secreted by the endometrium of the pregnant ewe. Peptide mapping of the two species of UTM-proteins demonstrated them to be structurally related. Furthermore, pulse-chase and continuous-labeling experiments indicated that both are produced from a common precursor of lower molecular weight. Purified UTM-proteins were found to be rich in basic amino acids, low in tyrosine, and apparently lacking in tryptophan. The proteins were about 5.6–5.7% carbohydrate by weight and bound the lectin, concanavalin A. UTM-proteins synthesized in vitro incorporated D-[3H]glucosamine. Analysis of [3H]glucosamine-labeled glycopeptides of Pronase-digested UTM-proteins by gel filtration indicated that most radioactivity is associated with one size class of oligosaccharide. UTM-proteins secreted by the endometrium in the presence of tunicamycin, an N-glycosylation inhibitor, were of lower molecular weight than those from control endometria, indicating that sugar chains are attached to the protein core via N-linkages to asparagine. UTM-proteins synthesized in culture incorporated [32P]orthophosphate, and tunicamycin inhibited this incorporation. Analysis of hydrolyzed UTM-proteins by paper chromatography indicated that much of the 32P was associated with mannose 6-phosphate. Because this moiety is the so-called lysosomal recognition marker and is present on uteroferrin, the acid phosphatase of porcine uterine secretions, we tested UTM-proteins for several enzymatic activities characteristic of lysosomes, but none was found. In conclusion, the UTM-proteins are related glycoproteins that, like porcine uteroferrin, contain mannose 6-phosphate, a result which suggests that secretion of glycoproteins with phosphorylated oligosaccharide chains may be a common feature of the progestational uterus.

INTRODUCTION

Progesterone of luteal or placental origin is necessary for the maintenance of pregnancy in mammals. One function of progesterone is to induce the secretion of endometrial proteins believed to be necessary for maintenance of pregnancy (see Aitken, 1979; Bazer et al., 1981; Bazer and First, 1983, for reviews). A variety of progesterone-induced polypeptides have been characterized, including proteins involved in nutrient transport (Adams et al., 1981; Buhi et al., 1982; Ducsay et al., 1984; McDowell et al., 1982), protease inhibitors (Mullins et al., 1980; Fazleabas et al., 1982) and lysosomal enzymes (R. M. Roberts et al., 1976; Roberts and Bazer, 1984; Hansen et al., 1985). In the ewe, the major proteins secreted by the endometrium in response to progesterone are a pair of basic glycoproteins with molecular weights ($M_r$) of 57,000 and 55,000 (Moffatt et al., 1987). These proteins, called the uterine milk proteins (UTM-proteins), are secreted by the lumenal and glandular epithelium of the endometrium throughout most of gestation.
While the function of the UTM-proteins has not been discovered, their abundance in uterine secretions suggest that they serve an important role during pregnancy. Gram quantities of the proteins can be recovered from the uterus of unilaterally pregnant ewes (Moffatt et al., 1987). Indeed, few if any other progesterone-induced proteins can be recovered in similar quantities. The UTM-proteins, therefore, have the potential to become a useful model for progesterone regulation of protein synthesis and secretion.

Characterization of the biochemical properties and biosynthesis of the UTM-proteins is important for several reasons. The two UTM-proteins have similar molecular weights and isoelectric points, which suggests that they are structurally related proteins. Their structural relationships to each other, however, have not been described. Similarly, it is not clear whether both proteins are synthesized and secreted independently or whether one is a product of the other. The structural differences between the two UTM-proteins must be established to identify differences that could be exploited to separate the proteins to determine whether they have similar biological functions. Finally, it has been of interest to determine whether the UTM-proteins, like uteroferrin of the pig uterus (Baumbach et al., 1984; Roberts and Bazer, 1984), possess phosphorylated oligosaccharide chains, a feature characteristic of many lysosomal hydrolases. If so, expression of this structural feature may be a common feature of many progesterone-induced glycoproteins and would be consistent with the hypothesis that the UTM-proteins represent hypersecreted lysosomal enzymes.

**MATERIALS AND METHODS**

**Materials**

Radioisotopes were purchased from Amersham (Arlington Heights, IL) and included L-[4,5-3H] leucine (45–70 Ci/mmol), D-[6-3H]glucosamine hydrochloride (10 Ci/mmol), D-[2-3H]mannose (10 Ci/mmol), Na-[35S] (carrier-free) and H332PO4 (carrier-free). D-[3H] mannose 6-phosphate was made from D-[2-3H] mannose by using adenosine 5'-triphosphate (ATP) and hexokinase according to the method of Rosen and Zeleznick (1966). IODO-GEN was obtained from Pierce Chemical Co. (Rockford, IL), and N-succinimidyl 3-(4-hydroxyphenyl) propionate (Bolton-Hunter reagent) was from Sigma Chemical Co. (St. Louis, MO).

Thin-layer chromatography plates (20 x 20 cm, precoated with 0.1 mm cellulose) were obtained from MCB reagents (Gibbstown, NJ). Fisher Scientific (Pittsburgh, PA) provided 2,5-diphenyloxazole (PPO), 2-iodoacetamide, and guanidine hydrochloride. Whatman (Clifton, NJ) provided chromatography paper and carboxymethyl cellulose (CM-cellulose). Bio-Gel P-4 was from Bio-Rad (Richmond, CA), and PD-10 columns were from Pharmacia (Piscataway, NJ). Tritiated oligosaccharide standards for calibration of the Bio-Gel P4 column were a gift from Dr. S. Kornfeld (Washington University, St. Louis, MO). Sigma supplied egg white lysozyme, bovine serum albumin, acid fuchsin, trypsin, tunicamycin, α-methyl-D-mannoside, D-glucose 6-phosphate, D-mannose N-tosyl lysine chloromethyl ketone 6-phosphate (TLCK)-treated chymotrypsin, concanavalin A (Con A), aprotonin, Nonidet P-40, and Pronase (non-specific protease from Streptomyces griseus). The Pronase was predigested before use according to the procedure of Varki and Kornfeld (1983). Phenyl-methylsulfonyl fluoride (PMSF) was from Eastman Chemicals (Rochester, NY). The cathepsin B substrate, Z-arg-arg-AMC, was obtained from Enzyme Systems (Livermore, CA), and other reagents for enzymatic assays were obtained as described by Hansen et al. (1985). Reagents for electrophoresis and tissue culture were as described by Basha et al. (1980).

**Endometrial Explant Cultures**

Intercaruncular endometria (300 mg in 3 ml medium or 500 mg in 15 ml medium) from pregnant ewes (Day 140) were cultured in a modified minimal essential medium (MEM) by using an explant system described by Moffatt et al. (1987). Cultures were carried out in the presence of various radioisotopes to radioactively label nascent proteins. Generally, media were prepared specifically depleted of the appropriate precursor. Cultures with L-[4,5-3H] leucine (50–100 μCi/dish) were done in MEM containing 0.10 or 0 times the usual leucine concentration. Cultures with D-[6-3H]glucosamine (50–200 μCi/dish) were sometimes done in MEM without glucose and with supplemental fructose (5 mg/ml), or with 0.25 times the usual glucose concentration and with supplemental L-glutamine at 5 mg/ml (Baumbach et al., 1984). To label phosphorylated proteins, endometrial tissue was cultured in phosphate-free MEM supplemented with H332PO4 (1 to 20 mCi/dish). Tissue was incubated in medium containing 5–20 μg/ml tunicamycin (added...
BIOCHEMICAL PROPERTIES OF THE UTERINE MILK PROTEINS

407

from a 10 mg/ml stock dissolved in dimethyl sulfoxide) to inhibit N-linked glycosylation (Tkacz and Lampen, 1975; Duksin et al., 1982).

Purification of UTM-Proteins

UTM-proteins from pooled uterine secretions of unilaterally pregnant ewes were purified as described by Moffatt et al. (1987). UTM-proteins appeared chemically pure by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE); after SDS-PAGE, the UTM-proteins were stained with Coomassie Blue R-250.

After incubation of endometrial tissue in the presence of D-[3H]glucosamine or H332PO4, radio-labeled UTM-proteins were isolated from culture medium by cation-exchange chromatography. Culture medium was dialysed against 10 mM Tris-HCl buffer; pH 8.2, and loaded onto a small (3.0 × 0.6 cm) column of CM-cellulose. After eluting unbound proteins with the above buffer, radiolabeled UTM-proteins were eluted with a 0 to 0.5 M NaCl gradient in 10 mM Tris, pH 8.2. The resulting preparation was composed of purified UTM-proteins as determined by one-dimensional SDS-PAGE (1-D PAGE), which was followed by fluorography.

Protein Determination

Protein concentration was usually determined by the method of Lowry et al. (1951), with bovine serum albumin as a standard. The method of Bradford (1976) was also employed with egg white lysozyme used as a standard. Column effluents were monitored by measuring absorbance at 280 nm. The accuracy of these methods in quantifying UTM-proteins was assessed by comparing estimates made on UTM-proteins dialysed extensively against double-distilled H2O with values determined gravimetrically.

Electrophoresis

One-dimensional SDS-PAGE was done by using the procedure of Laemmli (1970). Gels were 7.5 or 10% (w/v) polyacrylamide. Two-dimensional electrophoresis (2-D PAGE) was done as described by Roberts et al. (1984). Separation in the first dimension was by nonequilibrium pH gradient electrophoresis (NEPHGE), and separation in the second dimension was by SDS-PAGE using 10% (w/v) polyacrylamide gels. Radioactive proteins were detected by fluorography or autoradiography after the gels were dried (Roberts et al., 1984).

iodination

Because of their low tyrosine content, a batch of purified UTM-proteins was iodinated by a modification of the procedure of Bolton and Hunter (1973). Bolton-Hunter reagent was dissolved in toluene:ethyl acetate (1:1, v/v) to a concentration of 40 µg/ml. Forty microliters of this reagent were dried in a microcentrifuge tube, and then purified UTM-proteins (20 µg) and 0.1 M borate sodium, pH 8.5 (to bring volume to 40 µl) were added. The reaction was allowed to proceed for 30 min in an ice bath, and then transferred to a tube containing 10 µg of solid-phase IODO-GEN (Markwell and Fox, 1978) and 960 µl 0.02 M phosphate, pH 7.0, containing 0.4 M NaCl. Carrier-free Na[125I] (1 mCi) was added and reacted for 20 min at room temperature. Unreacted 125I was separated from radioiodinated UTM-proteins by chromatography on a Pharmacia PD-10 column. The specific activity of iodinated UTM-proteins ranged from 46 to 49 μCi/µg (n=2).

Peptide Mapping

The two isomers of UTM-proteins were separated from each other prior to peptide mapping by electrophoresis. 125I-labeled UTM-proteins were mixed with 50–100 µg nonradioactive UTM-proteins and an equal volume of 0.125 M Tris-HCl buffer, pH 6.8, containing 20% (w/v) sucrose, 10% (w/v) SDS and 5% (w/v) β-mercaptoethanol. After boiling for 3 min, samples were streaked across the length of the stacking gel of 7.5% or 10% (w/v) polyacrylamide gels and then separated by 1-D PAGE. Gels were stained with 0.125% (w/v) Coomassie Blue R-250, and bands corresponding to the two UTM-proteins were cut out separately. Proteins were eluted from the gel pieces by incubation overnight with 60% (v/v) acetic acid at 37°C. Samples were dialysed against double-distilled H2O and freeze-dried.

The two isomers of UTM-proteins were reduced and alkylated, and then digested with chymotrypsin as follows. The lyophilized samples were redissolved with 820 µl 6 M guanidine-HCl, pH 8.6, containing 2.5% (w/v) β-mercaptoethanol. After 4 h at room temperature, 50 mg solid 2-iodoacetamide was added and allowed to react for 20 min. After the addition of β-mercaptoethanol (100 µl), samples were dialysed extensively against double-distilled H2O, and 100 µl 1 M NH4OH, pH 8.0, and 5 µg (1 mg/ml) TLCK-treated chymotrypsin were then added. Samples were in-
cubated for 24 h at 37°C, boiled to inactivate chymotrypsin, and freeze-dried.

The iodinated peptides produced from this procedure were separated by using a modification of the method of Elder et al. (1977). Briefly, lyophilized samples were redissolved in 20–30 µl electrophoresis buffer [acetic acid:formic acid:water (15:5:80, v/v/v)] and then spotted onto 20 × 20-cm thin-layer cellulose plates. The peptides were first separated by flat-bed electrophoresis after the plates were sprayed with electrophoresis buffer. Separation was at 1 kV, and migration was monitored with a tracking dye of 1% (w/v) acid fuchsin. Separation in the second dimension was achieved by thin-layer chromatography using a solvent of 1-butanol:pyridine:acetic acid:water (32.5:25.5:20, v/v/v/v) that contained 7% (w/v) PPO. Iodinated peptides were detected by fluorography using Kodak XAR film.

Amino Acid Sequencing

A limited amino acid sequence of the amino termini of a mixture of one batch of purified UTM-proteins A and B (roughly 70–80% A) was determined with an Applied Biosystems Protein Sequencer (Foster City, CA) and a Beckman 890C spinning cup sequencer (Palo Alto, CA) with a 0.1 M quadrol program. The residues produced were converted to their phenylthiohydantoin derivatives and identified by high-performance liquid chromatography (HPLC).

Amino Acid Analysis

Proteins were hydrolyzed by means of 6 M HCl in sealed, evacuated tubes for 24 or 48 h at 105°C. Some samples were hydrolyzed in the presence of 0.35 M dimethyl sulfoxide to preserve cysteine residues. Analyses were performed on ninhydrin-derived samples by means of a Beckman Model 120C automated amino acid analyzer (Beckman Instr., Palo Alto, CA). As an alternate procedure, amino acids were derivatized with orthophthalaldehyde and separated on a reverse-phase C18 column by HPLC according to procedures recommended by Waters Associates of Milford, MA (bulletin on Waters HPLC systems for amino acid analysis). The two procedures were done with different batches of purified UTM-proteins and gave comparable results. Values were corrected for losses during hydrolysis by comparing amino acid ratios obtained at 24 and 48 h. Tryptophan was measured separately by the method of Basha and Roberts (1977).

Pulse-Chase and Continuous-Labeling Experiments

For the pulse-chase experiment, endometrial explants (500 mg) from a pregnant ewe (D 137) were cultured for 90 min in 15 ml MEM without leucine and for 30 min in 15 ml medium supplemented with 100 µCi L-[4,5-3H]leucine. Explants were then washed and incubated for 5 h in MEM without leucine. A single piece of tissue and a 1-ml aliquot of medium were collected at −1, 0, 10, 20, 30, 60, 90, and 300 min after removal of radiolabel. A continuous-labeling experiment was done similarly, except that explants were cultured continuously with L-[4,5-3H]leucine, and samples were collected at 15, 20, 30, 60, 90, and 330 min after addition of radio(label. The types of radiolabeled UTM-protein present in samples of medium and tissue samples were determined by immunoprecipitation. Medium was dialysed against distilled water, lyophilized, and reconstituted to the original volume with an immunoprecipitation buffer composed of 50 mM Tris-HCl, pH 7.5, containing 0.3 M NaCl, 2% (v/v) Nonidet P-40, 1 trypsin-inhibitory unit/l aprotonin, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM ethylenediamine-tetraacetic acid, and 0.02% (w/v) NaN₃. Samples of tissue were solubilized by sonication and freeze-thawing in 1 ml immunoprecipitation buffer. After centrifugation at 12,000 × g for 5 min, supernatant fractions were analyzed by immunoprecipitation. To immunoprecipitate the UTM-proteins, 50 µl of chicken antiserum to UTM-proteins (Moffatt et al., 1987) were added to 1 ml of sample, and the mixture was incubated overnight at 4°C. Immunoprecipitates were collected by centrifugation (12,000 × g for 5 min), washed three times with 10 mM Tris-HCl, pH 8.6, containing 0.6 M NaCl, 0.5% (v/v) Nonidet P-40, 0.1% (w/v) SDS, and 0.02% (w/v) NaN₃, washed once with distilled H₂O, and analyzed by 1-D PAGE and fluorography.

Carbohydrate Composition

Samples of purified UTM-protein were assayed for neutral sugars by the phenol sulfuric acid method (Dubois et al., 1956), for amino sugars by the procedure of Rondle and Morgan (1955), and for sialic acid by the resorcinol method (Svennerholm, 1958). Results were expressed on a weight basis as
grams of carbohydrate per gram of glycoprotein, with the protein contents being determined gravimetrically.

**Binding to Concanavalin A**

Binding of UTM-proteins to concanavalin A, a lectin that binds α-mannosyl and α-glucosyl residues (Krusius et al., 1976), was evaluated by a previously published method (Burridge, 1978; Roberts et al., 1984). Samples of uterine secretions (200 μg protein as determined by the method of Lowry) were resolved by 2-D PAGE. After electrophoresis, gels were equilibrated sequentially with acetic acid:ethanol:water (7:40:53 [v/v/v]) and 50 mM Tris-HCl, pH 7.5, containing 0.15 M NaCl and 0.02% (w/v) NaN₃. The gels were incubated for 16 h in the same buffer containing 2.1 μCi ¹²⁵I-labeled concanavalin A, iodinated by the IODO-GEN procedure (Markwell and Fox, 1978). After incubation, gels were thoroughly washed with buffer, dried, and exposed at −70°C to Kodak XRP-1 film to prepare autoradiographs.

**Gel Filtration of Glycopeptides**

 Supernatant samples of cultures of endometrial tissue with D-[6-³H]glucosamine were mixed with nonradioactive UTM-proteins; proteins were separated by SDS-PAGE, as described for peptide mapping, to produce separate preparations of [³H]glucosaminelabeled UTM-proteins A and B. After samples were extracted from the gel with 2 ml 60% (v/v) acetic acid and dialyzed against 10 mM Tris-HCl, pH 8.2, containing 0.2 M NaCl, 2.5 mg self-digested Pronase was added to each. The reaction proceeded for 48 h, with an additional 2.5 mg Pronase added at 24 h. The reaction was stopped by boiling. After removal of denatured Pronase by centrifugation, supernatants were loaded separately onto a 110 × 1.5-cm column of Bio-Gel P-4 that had been precalibrated with Man₉Glcitol[³H]Nac and Man₆Glcitol[³H]Nac standards (see Li et al., 1978; Kornfeld et al., 1978). Glycopeptides were eluted from the column by using 0.1 M acetic acid. Radioactivity in the resultant fractions (2.5 ml) was detected by liquid scintillation spectrometry. Acid hydrolysis of ³²P-labeled UTM-proteins were purified from culture medium by CM-cellulose chromatography, dialysed against double-distilled water and made 1 M with HCl. Samples were hydrolysed in sealed, evacuated tubes for 4 h at 100°C. After hydrolysis, samples were dried by rotary evaporation to remove acid, redissolved in H₂O, and spotted onto the top of a 15 × 45-cm Whatman No. 1 paper. Orthophosphate, D-mannose 6-phosphate and D-[2-³H]mannose 6-phosphate standards were spotted in separate lanes. Chromatography was performed for 21 h and used a solvent of pyridine:ethyl acetate:water:acetic acid (5:5:3:1, v/v/v/v). Appropriate lanes were cut into 1-cm strips, and radioactivity was measured by scintillation spectrometry. Phosphate was detected by spraying the chromatogram with molybdate spray (an aqueous solution of 1% [w/v] [NH₄]₆ MO₇O₂₄·4H₂O, 3% [w/w] perchloric acid and 0.1 N HCl) and developing it under ultraviolet light.

**Enzymatic Activity**

 Assays of purified UTM-proteins for glycosidase, arylsulphatase, and cathepsin D activities were performed as reported by Hansen et al. (1985). Cathepsin B activity was assayed as described elsewhere (Barrett and Kirschke, 1981). Proteolytic activity of UTM-proteins was also examined by using bovine casein suspended in 1% (w/v) agar plates (Bio-Rad Protease Substrate, see Bjerrum et al., 1975). Substrate tablets were dissolved separately in distilled water (final pH 7.2 in Tris-buffered saline), 0.10 M acetate buffer, pH 4.9, and 0.10 M glycine-HCl, pH 3.0. UTM-proteins (0.5 to 2.5 mg/ml) were
placed in wells cut in the agar and incubated for 48 h at room temperature. Protease activity was quantified as the diameter of the halo forming around each well. Trypsin (1 mg/ml) was used as a positive control.

RESULTS

Peptide Mapping

Peptide mapping was carried out on a batch of purified UTM-proteins to determine whether the 57,000 (A) and 55,000 (B) molecular-weight species of UTM-proteins were structurally related. As can be seen in Figure 1, the pattern of peptides produced from digestion of UTM-protein A was almost indistinguishable from the pattern produced from digestion of UTM-protein B. There was complete homology in the location and number of major peptide spots. While roughly equal amounts of radioactivity were loaded onto each plate, some of the quantitatively smaller peptide spots were more apparent for UTM-protein A than for UTM-protein B.

FIG. 1. Peptide maps of UTM-proteins A and B. Peptides formed from digestion with TLCK-treated chymotrypsin were separated by electrophoresis (x-axis) and thin-layer chromatography (y-axis) and localized by autoradiography. Note that the patterns of peptides produced after digestion of UTM-protein A was homologous to the pattern of peptides produced after digestion of UTM-protein B.
Almost complete homology between A and B forms from another batch of purified UTM-proteins were also observed after digestion with chymotrypsin or cyanogen bromide and analysis of the peptides by 1-D PAGE (results not shown). Because of the high degree of similarity in peptide maps, the two forms of UTM-proteins likely have similar structures.

Amino Acid Sequencing

The N-terminal amino acid sequence of a mixture of UTM-proteins A and B was determined by using two procedures for automated amino acid sequence measurements. The sequences derived from the two methods gave identical results (Table 1). With the vapor-phase, Applied Biosystems procedure, a minor secondary signal was detected for residues 1 and 3, but thereafter only a single amino acid was deleted. The presence of only one series of primary signals is indicative of extensive homology in the N-terminal amino acid sequence of UTM-proteins A and B. If the proteins had been dissimilar, a consistent secondary signal would have been observed throughout the sequence.

Precursor-Products Relationships

The biosynthesis of the UTM-proteins was examined by using pulse-chase and continuous-labeling techniques to determine whether the proteins are derived from a common precursor or whether one isomer of the UTM-proteins is a precursor of the other. In the pulse-chase experiment, endometrial tissue from a ewe of Day 137 of pregnancy was cultured for 30 min with [3H]leucine. UTM-proteins in culture medium and in tissue at times after removal of radioisotope were analyzed by immunoprecipitation and 1-D PAGE. The only immunoprecipitable proteins secreted into medium were UTM-proteins A and B (results not shown). These first appeared simultaneously at 90 min after removal of [3H]leucine. In endometrial tissue, however, the first immunoprecipitable protein to be detected (at 20 min) was a single protein having an Mr ~ 54,000 (Fig. 2). Subsequently, this protein disappeared and was replaced by the 55,000 and 57,000 molecular weight UTM-proteins A and B. Similar results were observed in an experiment in which endometrium was cultured continuously with [3H]leucine (Fig. 2). The only immunoprecipitable proteins in medium were UTM-proteins A and B, but in tissue an immunoprecipitable protein of Mr ~ 54,000 preceeded the appearance of UTM-protein A and UTM-protein B.

Optical Properties and Weight Estimates

Because peptide mapping, labeling, and sequencing experiments indicated UTM-proteins A and B were closely related polypeptides, and because they could not be separated by methods other than electrophoresis, further characterization of the UTM-proteins was carried out by using a mixture of the two isomers. Purified preparations of UTM-proteins did not exhibit an absorption maximum at 280 nm (Fig. 3), a result which indicated that the proteins were probably devoid of tryptophan. Absorption peaks were detected at 255–260 nm, with a shoulder at 270–275 nm. These peaks were probably due to the presence of phenylalanine and tyrosine, respectively.

Based on repeated (n=4) dry weight estimates of a batch of UTM-protein, a 1 mg/ml solution of UTM-proteins gave values of 0.92 mg protein/ml by the method of Lowry et al. (1951) and 1.49 mg/ml by Bradford’s (1976) method. The E₂₈₀ value for UTM-proteins (0.97 cm⁻¹) was lower than for most proteins, presumably because the proteins lacked tryptophan.

<table>
<thead>
<tr>
<th>Position no.</th>
<th>Primary signal</th>
<th>Secondary signal</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Glu (79%)</td>
<td>Gly (21%)</td>
</tr>
<tr>
<td>2</td>
<td>Lys</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Gln (94%)</td>
<td>Phe (6%)</td>
</tr>
<tr>
<td>4</td>
<td>Gln</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>(His)</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Ser</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Gln</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Gln</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>His</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>Ala</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>Asn</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>Leu</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>Val</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>Leu</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>Leu</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>Lys</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>Lys</td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>Ile</td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>Ala</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>Ala</td>
<td></td>
</tr>
</tbody>
</table>

* Determined from a purified mixture of UTM-proteins by using an Applied Biosystems Protein Sequencer. Primary signals were identical to those shown here when a Beckman Spinning cup sequencer was used. The amino acid in parentheses denotes a questionable cell.
FIG. 2. Pulse-chase and continuous labeling of UTM-proteins present in endometrial tissue. [3H]leucine-labeled UTM-proteins were immunoprecipitated from homogenized endometrium, separated by 1-D PAGE using 7.5% (w/v) polyacrylamide gels and localized by fluorography. No radiolabeled proteins were present when preimmune serum was used instead of antiserum (results not shown). In pulse-chase experiments, the first protein to appear in tissue was a 54,000 Mₐ protein, which subsequently was replaced by UTM-protein A and B (57,000 and 55,000, respectively). Similar results were found in continuous-labeling experiments, except that after its initial appearance at 20 min, the precursor was present throughout the sampling period.

Amino Acid and Carbohydrate Composition

Results of amino acid analysis are presented in Table 2. The UTM-proteins were found to be high in leucine, lysine, and threonine but contained only two tyrosine residues per molecule. Based on the lack of an absorption peak at 280 nm (Fig. 3) and the low values measured colorimetrically (Table 2), tryptophan appeared to be absent. Based on determinations on one batch of purified UTM-proteins done at three concentrations, the carbohydrate content was estimated as 5.6% by weight and consisted of 2.8% neutral sugars, 2.5% amino sugars, and 0.3% sialic acid. Assuming a carbohydrate composition of 5.6%, the molecular weight of UTM-proteins as estimated by amino acid analysis was 53,700.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Molar percent</th>
<th>Residues/molecule</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asx</td>
<td>9.4</td>
<td>42</td>
</tr>
<tr>
<td>Glx</td>
<td>11.0</td>
<td>49</td>
</tr>
<tr>
<td>His</td>
<td>5.5</td>
<td>24</td>
</tr>
<tr>
<td>Ser</td>
<td>3.1</td>
<td>14</td>
</tr>
<tr>
<td>Arg</td>
<td>2.9</td>
<td>13</td>
</tr>
<tr>
<td>Gly</td>
<td>1.9</td>
<td>8</td>
</tr>
<tr>
<td>Thr</td>
<td>8.4</td>
<td>37</td>
</tr>
<tr>
<td>Ala</td>
<td>6.9</td>
<td>31</td>
</tr>
<tr>
<td>Tyr</td>
<td>0.5</td>
<td>2</td>
</tr>
<tr>
<td>Met</td>
<td>3.5</td>
<td>16</td>
</tr>
<tr>
<td>Val</td>
<td>5.1</td>
<td>23</td>
</tr>
<tr>
<td>Phe</td>
<td>5.7</td>
<td>25</td>
</tr>
<tr>
<td>Ile</td>
<td>5.6</td>
<td>25</td>
</tr>
<tr>
<td>Leu</td>
<td>15.3</td>
<td>68</td>
</tr>
<tr>
<td>Lys</td>
<td>10.0</td>
<td>44</td>
</tr>
<tr>
<td>Pro</td>
<td>4.3</td>
<td>19</td>
</tr>
<tr>
<td>Half-Cys</td>
<td>1.0</td>
<td>4</td>
</tr>
<tr>
<td>Trp</td>
<td>0.1</td>
<td>0</td>
</tr>
</tbody>
</table>

\( ^{a} \)Values for Asx to Lys are the average of two separate determinations made by means of the o-phthalaldehyde derivatization method. Proline and half-cystine residues were measured after ninhydrin derivatization on a Beckman amino acid analyzer. Tryptophan was measured colorimetrically by the method of Basha and Roberts (1977).

\( ^{b} \)Calculations were based on the assumption of 2 tyrosine residues per molecule. The calculated molecular weight, based on the amino acid composition presented in this column and a carbohydrate composition of 5.6% by weight, is 53,700.

---

Incorporation of Radiolabeled Sugars

Endometria from two pregnant ewes were cultured separately in the presence of D-\(^{3}\)H glucosamine. After 24 h of culture, proteins secreted into culture medium were resolved by 2-D PAGE. As indicated by fluorographs of the dried gels (Fig. 4), \(^{3}\)H glucosamine was incorporated into UTM-proteins.

Endometria from four ewes were also cultured with \(^{3}\)H leucine in the presence or absence of tunicamycin, an inhibitor of N-linked glycosylation of proteins (Tkacz and Lampen, 1975). Radiolabeled proteins released into medium were analysed by 1-D PAGE and fluorography (Fig. 5). In all cases, UTM-proteins secreted by endometrium treated with tunicamycin were of lower molecular weight than those secreted by nontreated endometrium. The shift in molecular weight was about 3000. Two forms of UTM-proteins were secreted by tunicamycin-treated endometrium.
tissue. These had molecular weights of approximately 52,000 and 54,000, respectively.

**Binding to Concanavalin A**

Binding of UTM-proteins to Con A was determined by incubating 2-D electrophoretograms of uterine secretions from a pregnant ewe with \(^{125}\text{I}\)-labeled Con A. After washing out unbound radioactivity, Con A that bound to proteins in the gel was localized by autoradiography of the dried gel. As shown in Figure 6, there was binding of \(^{125}\text{I}\)-labeled Con A to UTM-proteins. Binding could be prevented by the addition of \(\alpha\)-methyl-D-mannoside, a competing ligand that binds to Con A (results not shown). Iodinated Con A also bound to purified UTM-proteins transferred to nitrocellulose by Western blotting (results not shown).

**FIG. 5.** Analysis of \(^{3}\text{H}\)-leucine-labeled secretory products of endometrium produced in the presence of tunicamycin. Endometrium (500 mg) was cultured for 24 h in MEM containing 0.1 times the usual leucine concentration supplemented with 100 \(\mu\text{Ci}\) \(\text{L-[4,5}^{3}\text{H}\text{] leucine and with or without the N-glycosylation inhibitor, tunicamycin (5 m}\mu/l\). Radiolabeled proteins present in medium at the end of culture were dialysed (Mr cutoff = 12,000–14,000), separated by 1-D PAGE (7.5% polyacrylamide gels) and localized by fluorography. The major radiolabeled proteins secreted were the UTM-proteins. Note that the Mr of these proteins from tunicamycin-treated cultures was about 3000 less than from control cultures, and that two forms of UTM-proteins were produced in both types of culture.

**FIG. 6.** Binding of \(^{125}\text{I}\)-labeled concanavalin A to 2-D PAGE electrophoretograms of uterine proteins from Day 120 of pregnancy (200 \(\mu\text{g}\) as measured by Lowry assay). Shown here is an autoradiogram of the gel after incubation with \(^{125}\text{I}\)-Con A and extensive washing. Several proteins bound Con A, including the UTM-proteins (arrow).

**FIG. 7.** Resolution of \(^{3}\text{H}\text{glucosamine-labeled glycopeptides of UTM-protein A (top panel) and UTM-protein B (bottom panel) by gel filtration. Radiolabeled UTM-proteins were purified from medium of endometrium cultured with D-[6-\(^{3}\text{H}\text{] glucosamine. Glycopeptides were produced by Pronase digestion and resolved by gel filtration using Bio-Gel P-4. The arrow labeled "GM\(_9\)" represents the elution volume of a Man\(_9\) Glcitol[\(^{3}\text{H}\text{] NAc standard.}})
Gel Filtration of Glycopeptides

Endometrium from a pregnant ewe at Day 140 of pregnancy was cultured in the presence of [3H]glucosamine. [3H]glucosamine-labeled UTM-proteins A and B were then purified separately from the culture medium by SDS-PAGE and digested with Pronase to individual amino acids and glycopeptides. The products of digestion were analysed by gel filtration using Bio Gel P-4 to determine size and minimum number of oligosaccharide chains on the UTM-proteins (Fig. 7). For both forms, a major peak of [3H]glucosamine eluted ahead of the Man9-Glcitol[3H]NAc standard. Another quantitatively smaller peak of lower molecular weight was detected for UTM-protein A. For glycopeptides of UTM-protein B, this second peak was present as a shoulder of the major peak of glycopeptide. Glycopeptides produced by digestion of a mixture of [3H]glucosaminelabeled UTM-proteins A and B derived from another ewe produced a pattern similar to those of Figure 7 (results not shown). Because of the similarities in elution profiles of the glycopeptides, the carbohydrate portion of UTM-protein A appeared substantially similar to that of UTM-protein B.

Immuno precipitation of 32P-labeled UTM-Proteins

To determine if newly synthesized UTM-proteins were phosphorylated, endometrial cultures of three ewes were done in the presence of H332P04. Radio-labeled UTM-proteins were immunoprecipitated from culture medium and analysed by 1-D PAGE and fluorography. As illustrated in Figure 8, UTM-proteins from each culture were phosphorylated. Incorporation of 32P into UTM-proteins could be inhibited by tunicamycin, which indicates that 32P was present on the oligosaccharide portion of the molecule.

Acid Hydrolysis of 32P-Labeled UTM-Proteins

To evaluate if the phosphate associated with UTM-proteins was present as mannose 6-phosphate, UTM-proteins labeled with 32P were purified from culture medium of a Day 140-pregnant ewe and hydrolysed under conditions that would hydrolyse most glycosidic bonds to yield monosaccharides. The phosphoester bond to carbon 6 of D-mannose is, however, stable under these conditions (Distler et al., 1979). The hydrolysate was subjected to descending paper chromatography, and the migration of 32P in the hydrolysate was compared to migration of a D-mannose 6-phosphate standard (Fig. 9). Two major peaks of 32P were resolved, each comprising roughly 50% of the radioactivity resolved. One peak comigrated with D-mannose 6-phosphate, while the other peak migrated with inorganic phosphate. Similar analysis of 32P-labeled UTM-proteins purified from culture medium of another pregnant ewe gave similar results (data not shown).

Enzymatic Activity

Purified UTM-proteins were tested for an array of enzymic activities characteristic of lysosomal enzymes. No activity was found when tested with substrates for β-N-acetylglucosaminidase, α-galactosidase, β-galactosidase, β-glucosidase, β-glucuronidase and α-mannosidase. Similarly, the UTM-proteins did not exhibit arylsulphatase, cathepsin B, or cathepsin D activities and did not digest casein in an agar plate assay carried out at pH 3.0, 4.9, or 7.2.

FIG. 8. Analysis of 32P-labeled UTM-proteins by immunoprecipitation and 1-D PAGE. Endometrium (300 mg) was cultured with 60 μCi L-[3,5-3H]leucine or 11 mCi H332P04 in 3 ml leucine-free or phosphate-free MEM. Cultures were done with 0 or 20 μg/ml tunicamycin. After 24 h of culture, 1-D PAGE (10% polyacrylamide) and fluorography were used to analyse the total array of non-dialysable secretory products (Mₚ cutoff = 12,000–14,000) or UTM-proteins immunoprecipitated from medium (IMP). Note that many proteins were phosphorylated, including UTM-proteins. Tunicamycin caused a decrease in the molecular weight of UTM-proteins (compare lanes 5 and 6) and prevented phosphorylation of UTM-proteins (compare lanes 7 and 8).
FIG. 9. Analysis of the acid hydrolysate of \(^{32}\text{P}\)-labeled UTM-proteins by descending paper chromatography. Bars represent the areas of migration of nonradioactive mannose 6-phosphate (M-6-P) and orthophosphate (P) standards. Migration of \(^{3}\text{H}\)mannose 6-phosphate is indicated by the solid line. Two peaks of \(^{32}\text{P}\) in the UTM-protein hydrolysate were resolved (dotted line). One peak comigrated with \(^{3}\text{H}\)mannose 6-phosphate and mannose 6-phosphate standards, while the other peak migrated with inorganic phosphate. The peak of tritiated material at 10 cm represents mannose.

DISCUSSION

Results indicate that the two major proteins secreted by the endometrium of sheep from Day 30 of pregnancy to term (Moffatt et al., 1987) are structurally related glycoproteins. These two polypeptides, called the uterine milk proteins, have similar isoelectric points and differ only slightly in molecular weight, as determined by SDS-PAGE. Each gives rise to a similar spectrum of peptides when cleaved by chymotrypsin. Limited N-terminal sequencing of the purified mixture of the two polypeptides revealed little evidence for heterogeneity in amino acid sequence. Together, these data provide convincing evidence that the UTM-proteins are similar in structure. Moreover, both pulse-chase and continuous-labeling experiments revealed that both UTM-proteins are formed intracellularly from a common precursor of lower molecular weight. Both secreted polypeptides are glycosylated as demonstrated by results presented here and in the companion paper (Moffatt et al., 1987). They stain with the periodic acid-Schiff reagent, bind concanavalin A, and incorporate \(^{3}\text{H}\)glucosamine when synthesized in vitro.

The UTM-proteins are unusual in that they lack tryptophan, and consequently show no absorption maximum at 280 nm. As a result, assessing the amount of protein by measuring absorbance at 280 nm can result in a gross underestimate of UTM-protein content. Their tyrosine content is also low. They possess a high content of leucine and are rich in threonine. Like uteroferrin and the other major progesterone-induced components of the pig uterus, they are strongly basic and contain large amounts of lysine, a feature that may contribute to their ability to bind the overlying placenta and to their solubility.

Data reported here are consistent with the UTM-proteins having one or possibly two oligosaccharide chains per molecule. Together, the UTM-proteins are about 5.6% carbohydrate by weight, which indicates the likely presence of 14–17 monosaccharide units per molecule. Furthermore, a major peak of radio-
activity with a size greater than a ten-unit, branched oligosaccharide standard was resolved when [3H]-glucosamine-labeled glycopeptides were separated by gel filtration. This carbohydrate appears to be largely in the form of N-linked chains since tunicamycin reduced the apparent molecular weight of secreted UTM-proteins, presumably by inhibiting N glycosylation. About one-half of the carbohydrate was in the form of neutral sugars, and most of the remainder was amino sugars. Less than one residue of sialic acid per molecule of protein was detected. These results indicate that the oligosaccharide chains were at least partially processed to more complex types (Kornfeld and Kornfeld, 1976; Kornfeld, 1982).

Cultured explants of endometrium incorporated H_3^{32}PO_4 into both UTM-proteins. This labeling pattern was inhibited by tunicamycin, a result which suggests that the ^32P was incorporated predominately into the carbohydrate portion of the molecule. Analysis of acid-hydrolysed UTM-proteins indicated that at least some of this ^32P was present as mannose 6-phosphate, the so-called lysosomal recognition marker (Shepherd et al., 1983). The remainder of the incorporated phosphate migrated as inorganic phosphate, and this phosphate could have been formed either from hydrolysis of other sugar phosphate linkages or from partial degradation of mannose 6-phosphate.

It has previously been shown that uteroferrin, the major progesterone-induced glycoprotein of the pig uterus, is also secreted carrying mannose 6-phosphate (Baumbach et al., 1984). Indeed, many of the properties of uteroferrin, including its acid phosphatase activity, are characteristic of a lysosomal acid hydrolase (Roberts and Bazer, 1984). The possibility has been raised that during the hypersecretory state induced in the endometrium by progesterone, uteroferrin is diverted away from lysosomes to the cell exterior as a secretory product. These findings, together with the fact that uterine secretions of the ewe and other species contain other lysosomal enzymes (G. P. Roberts and Parker, 1974; G. P. Roberts et al., 1976a,b; R. M. Roberts et al., 1976; Hansen et al., 1985) suggest that the UTM-proteins may also possess some as yet undefined acid hydrolase activity.

It is not yet known why the UTM-proteins differ in apparent molecular weight. The difference in electrophoretic mobility of the two is consistent with a molecular weight difference of about 2000. It is possible, therefore, that the glycoproteins differ in the number or degree of complexity of their carbohydrate chains. Experiments with tunicamycin demonstrated the presence of a pair of lower molecular weight molecules. Since tunicamycin inhibition of glycosylation may not have been complete, these products could represent a completely aglycosylated form (Mr ~ 52,000) and a form bearing a single oligosaccharide chain (Mr ~ 54,000). The pattern of glycopeptides formed by Pronase digestion of the A and B proteins appeared similar as determined by separation on Bio-Gel P-4. One possibility is that UTM-protein A has two oligosaccharide chains, while UTM-protein B has only one. Further experimentation is needed to understand the metabolic and structural relationships between the two forms.

ACKNOWLEDGMENTS

The authors thank Dr. Stuart Kornfeld, Washington University, St. Louis, MO, for donation of oligosaccharide standards; and Dr. Ned Siegel, Div. of Biol. Sci., Monsanto Co., St. Louis, MO, and Dr. Ben Dunn, University of Florida, for performing amino acid sequencing. We are also grateful to G. von Mering, M. Hoggard, C. Ketcham, A. Seassera and W. Clark for technical assistance, and to S. Sams for typing the manuscript.

REFERENCES
