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Purification, Secretion and Immunocytochemical Localization of the Uterine Milk Proteins, Major Progesterone-Induced Proteins in Uterine Secretions of the Sheep[1]

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ABSTRACT

Restriction of the conceptus to one uterine horn of the pregnant ewe results in the accumulation of fluid called uterine milk (UTM) in the contralateral horn. Two basic polypeptides, called the uterine milk proteins (UTM-proteins; M_r = 55,000 and 57,000 as determined by polyacrylamide-gel electrophoresis using sodium dodecyl sulfate), accounted for the majority of the protein in uterine milk. The two UTM-proteins were glycoproteins and were readily purified from uterine fluids by cation-exchange chromatography on carboxymethyl (CM)-cellulose followed by Sephacryl S-200 gel-filtration. The purified UIM-proteins had a weight-average molecular weight of 50,700 ± 4,200, as determined by equilibrium sedimentation analysis. Endometrial explants from pregnant ewes were cultured in the presence of radioactive amino acids and released UTM-proteins into the medium as their major secretory products. The UTM-proteins were secreted into the uterine lumen of nonpregnant, ovariectomized ewes given daily injections of progesterone. Estrone alone was ineffective in inducing UTM-protein production. Immunocytochemical studies indicated that synthesis of the UTM-proteins was confined to the surface and glandular epithelium of the uterus.

INTRODUCTION

The uterine endometrium is a hormonally responsive exocrine tissue, a major function of which is to provide an appropriate environment for the developing mammalian conceptus during pregnancy. There have been numerous descriptive studies of proteins secreted by the endometrium of animals during pregnancy and in response to gonadal steroids (for a review, see Aitken, 1979). Particular attention has been paid to secretions produced by those animals in which the trophoblast is noninvasive or in which firm attachment between the developing placenta and uterus occurs fairly late in pregnancy. In such species, the conceptus is believed to have a more extensive and prolonged dependence upon secretions of the endometrium for nutritional support (Bazer et al., 1981; Bazer and Roberts, 1983). Ectopic pregnancies in such animals are also unknown. The pig provides an excellent example of a species with a noninvasive type of placentation of the diffuse, epitheliochorial type. Its uterus produces copious quantities of secretory products in response to progesterone (Roberts and Bazer, 1980). Indeed, uterine secretions of the pig have been suggested to act as a complex culture medium for the loosely attached conceptus (Bazer et al., 1981). Additionally, proteins in the pregnant uterus may play roles other than in nutrition. A family of protease inhibitors in pig uterine secretions has been described (Mullins et al., 1980; Fazleabas et al., 1982) that may protect the endometrium from proteases released by the conceptus. Other functions of uterine secretions include roles in growth regulation (Heap et al., 1982) and immunoprotection (Murray et al., 1978).

The sheep, like the pig, possesses an epitheliochorial type of placenta. In this species, however, limited erosion of the uterine epithelium and its subsequent

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replacement begin to occur after Day 20 of pregnancy (King et al., 1982) as the placentomes begin to form. These structures result from the conjunction of placental cotyledons and maternal caruncles and are the major sites of gas and micronutrient exchange between the mother and conceptus. The intercaruncular regions of the endometrium, however, remain glandular, and secretions produced by the uterine glands have been called uterine milk because of their presumed nutritional role (Amoroso, 1952). Studies on the composition of uterine milk have been difficult to perform because only small volumes of fluid can be recovered from the pregnant uterus. However, if the conceptus is surgically restricted to one horn of the ewe's bicornuate uterus (Bazer et al., 1979), or if a pouch unoccupied by placental tissue is created (Harrison et al., 1976), uterine fluid accumulates and can be collected easily. Presumably this nongravid portion of the uterus responds to the hormones of pregnancy in much the same manner as the region occupied by the conceptus. Fluid accumulates because the secretions are not absorbed by the placenta, and this provides a rich source of material for analysis.

The major proteins present in the ligated horn of unilaterally pregnant ewes are a pair of proteins having molecular weights (M_r) of about 57,000 and 55,000 (Bazer et al., 1979). In this paper, we describe methods of purification of these polypeptides, called the uterine milk-proteins (UTM-proteins), and provide evidence that they are products of the surface and glandular epithelium of the endometrium whose secretion is induced by progesterone.

MATERIALS AND METHODS

Materials

Materials used for tissue culture and polyacrylamide gel electrophoresis (PAGE) have been described elsewhere (Basha et al., 1980). Concentrations of leucine and methionine in minimal essential medium (MEM) were maintained at 0.1 times the normal amount when those amino acids were used as radiolabeled substances. Amersham (Arlington Heights, IL) provided L-[^{35}S] methionine (spec. act. = 810 Ci/mmol), and Schwarz-Mann (Cambridge, MA) supplied L-[4,5-^{3}H] leucine (spec. act. = 60–62 Ci/mmol).

Whatman (Clifton, NJ) was the source of carboxymethyl cellulose (CM-cellulose). Estrone, progesterone, molecular weight standards, and bovine thyroglobulin were from Sigma Chemical Co. (St. Louis, MO). Morphocarbodiimide was from Aldrich Chemical Co. (Milwaukee, WI), Vectastain ABC immunoperoxidase–staining kit was from Vector Laboratories (Burlingame, CA), rabbit anti-chicken immunoglobulin (Ig G was from U.S. Biochemicals (Cleveland, OH), and 3-amino-9-ethylcarbazole was from Sigma Chemical Co.

Animals and Collection of Uterine Proteins

Animals used were multiparous ewes, primarily of Rambouillet and "Florida Native" breeding. Ewes were checked daily for estrous behavior by using vasectomized rams. Only those animals exhibiting at least two estrous cycles of 16–18 days duration were used.

Twenty-three ewes were bred twice daily, by natural mating, from the onset of estrus (Day 0) until the cessation of sexual receptivity. On Day 5, the uterine horn contralateral to the ovary bearing the corpus luteum was ligated (Bazer et al., 1979) to create a nongravid, ligated uterine horn and confine the conceptus to the remaining nonligated uterine body and horn. Uterine secretions and endometrium for explant culture were obtained aseptically from ewes on Days 30 (n=2), 60 (n=5), 90 (n=4), 120 (n=5), 136 (n=2), 140 (n=2), or 144 (n=3) of gestation. Uterine fluids were obtained by aspirating the ligated uterine horn (Bazer et al., 1979). When no fluid was visible, the ligated horn was flushed in situ with 5 or 10 ml of sterile 0.9% (w/v) NaCl, and flushings were recovered by aspiration through an oviductal cathether. Endometrial samples for explant culture were obtained from both ligated and gravid uterine horns.

A preliminary experiment was done to determine whether secretion of the UTM-proteins could be induced by gonadal steroids. Two cyclic ewes were subjected to midventral laparotomy during which one randomly chosen uterine horn was ligated and both ovaries were removed. After 30 days post-ovariectomy, ewes received daily s.c. injections of progesterone (50 mg) and estrone (5 μg). This daily dosage of progesterone has been shown to be adequate for maintenance of pregnancy (Foote et al., 1957; Alexander and Williams, 1966). After 120 days of treatment, uterine secretions and endometrium were obtained. Uterine secretions were analysed by two-dimensional PAGE for detection of the UTM-proteins. Endometrium was cultured as described later to determine if UTM-proteins were produced in vitro.

In another experiment designed to more closely examine steroidal induction of the UTM-proteins, 12 ovariectomized ewes with unilateral uterine ligations received daily injections of 5 μg estrone, 50 mg progesterone, 5 μg estrone plus 50 mg progesterone, or the injection vehicle (1 ml of corn oil; ethanol, 9:1, v/v) for 30 days. Uterine secretions and endometrium were obtained on Day 31 after initiation of injections and were treated similarly to the samples from the ewes treated for 120 days.

Seven cycling ewes were used for collection of uterine secretions on Days 0, 6, 8, 10, 12, 14, and 16 of the estrous cycle. Each uterine horn was flushed with 5–10 ml sterile saline (0.9%, w/v). The flushings were recovered by means of an oviductal catheter and frozen at −20°C until analysed by two-dimensional PAGE.

Purification of Uterine Milk Proteins

The high isoelectric point of the major proteins in uterine secretions facilitated their purification. All separation procedures were carried out at 4°C. Uterine secretions containing 10–75 mg protein were extensively dialyzed (three changes of 4 l each) against Tris-HCl buffer (10 mM Tris-HCl, pH 8.2, containing 0.02% NaN_3, w/v) and loaded onto a 10 × 0.9-cm column of CM-cellulose, preequilibrated in Tris-HCl buffer. After eluting acidic proteins by washing the column with buffer, basic proteins were eluted with either 0.5 M NaCl in Tris-HCl buffer or with a linear gradient (200 ml) of NaCl (0 to 0.5 M) in the same buffer. Linearity of the salt gradient was monitored by conductivity measurements of column effluents using a conductivity bridge (Yellow Springs Inst., Yellow Springs, OH). The last peak of material eluting from the column in the salt gradient (Peak 3) contained primarily UTM-proteins. The eluted proteins were dialyzed against Tris-HCl buffer containing 0.33 M NaCl, and 20-mg aliquots were then chromatographed on a 85 × 1.5 cm Sephacryl S-200 column. The column was preequilibrated with the same buffer and calibrated with ribonuclease A (M_r = 13,700), chymotrypsinogen (M_r = 25,600), uteroferrin (M_r = 35,000), ovalbumin (M_r = 45,000), bovine serum albumin (M_r 69,000), transferrin (M_r = 77,000), and aldolase (M_r = 158,000). Column fractions were monitored for protein by absorbance at 280 nm and by the method of Lowry et al. (1951), and for neutral sugars by the phenol-sulfuric acid method (Dubois et al., 1956).

Large-scale purifications were carried out as follows. Dialyzed uterine fluid (200–300 ml) was mixed with a slurry of CM-cellulose (50–60 ml) in 0.01 M Tris-HCl buffer, pH 8.2, for 20–30 min by using slow stirring. The CM-cellulose was then filtered on a sintered glass funnel and washed thoroughly with Tris-HCl buffer. Basic proteins were eluted by resuspending the CM-cellulose with 0.5 NaCl in Tris-HCl buffer and saving the filtrate that contained the UTM-proteins. This material was fractionated by gel filtration on a 82.5 × 4.2-cm Sephadex G-100 column in the presence of 10 mM acetate buffer, pH 4.9, containing 0.33 M NaCl.

Endometrial Explant Cultures

Intercaruncular endometrium was cultured as described by Basha et al. (1980). Briefly, 500 mg of 1 mm^3 explants were cultured in 15 ml of a specially modified MEM (Godkin et al., 1982) containing 5–50 μCi of L-[4,5-^{3}H] leucine or L-[^{35}S] methionine for 24 h at 37°C in an atmosphere of 50% N_2, 47.5% O_2, and 2.5% CO_2 (v/v). Incubations were terminated by centrifugation (20,000 × *g*, 4°C, 15 min), and decanting of the medium. The culture medium was extensively dialyzed (6000–8000 molecular weight-cutoff tubing) against Tris-HCl buffer to remove unicorporated radiolabel. The volume and radioactivity of culture medium after dialysis was measured to calculate the amount of radiolabel incorporated into nondialyzable secretory macromolecules. Tissue was solubilized as described by Godkin et al. (1982).

Protein Determination

Protein concentrations of dialyzed uterine fluids and flushings were determined by the method of Lowry et al. (1951) with bovine serum albumin as a standard. Column effluents were monitored as above, or by monitoring absorption at 280 nm. The method of Bradford (1976) was also employed to measure protein content of some samples of purified UTM-proteins with egg white lysozyme or human IgG used for standards.

Electrophoresis

One-dimensional PAGE in the presence of sodium dodecyl sulfate (SDS-PAGE) was performed as described by Laemmli (1970). Two-dimensional PAGE was done as described by Roberts et al. (1984). Acidic proteins (pI<8) were resolved in the first

dimension by isoelectric focusing while basic proteins were separated by nonequilibrium pH gradient electrophoresis (NEPHGE). Separation of both acidic and basic proteins in the second dimension was by electrophoresis in 10% (w/v) gels. Procedures for localizing proteins and glycoproteins by Coomassie Blue, silver and periodic acid-Schiff staining and by fluorography have been reported elsewhere (Roberts et al., 1984).

Equilibrium Sedimentation

Analyses were performed using a six-channel Yphantis centerpiece in a Beckman-Spinco Model E ultracentrifuge with Rayleigh interference optics. Centrifugation lasted 16–20 h at 20,410 rpm and 20°C for solubilized clot proteins, and at 15,220 rpm and 25°C over the same time period for purified uterine milk proteins. Calculations of weight-average molecular weights were made according to Yphantis (1964), assuming a partial specific volume for the proteins of 0.72 ml/g.

Antibody Production

Antisera to purified UTM-proteins coupled to bovine thyroglobulin were generated in mature roosters. Morphocarbodiimide was used to conjugate the antigens (Goodfriend et al., 1966). The molar ratios of UTM-proteins:thyroglobulin:morphocarbodiimide were 10:1:10000 (in 1 ml). After 16 h of gentle agitation, conjugated proteins were isolated by gel filtration. Roosters received three s.c. injections of immunogen (1 mg) in Freunds' complete adjuvant at 3-wk intervals. Subsequent immunizations (0.5 mg) were given at 3-wk intervals in incomplete adjuvant. Roosters were bled 10–14 days after each immunization. Antibody titers and specificities were evaluated by immunization. Antibody titers and specificities were evaluated by immunodiffusion (Ouchterlony and Nilsson, 1973) in plates of 1% (w/v) Noble agar in phosphate-buffered saline (PBS).

Immunocytochemistry

Endometria collected from pregnant ewes at Day 60–140 of pregnancy were fixed for 16 h at 4°C in Bouin's solution, washed for 24 h in 50% (v/v) ethanol, and dehydrated in a graded series of ethanol (50–100%) and 100% xylene prior to embedding in paraffin (Galigher and Kozloff, 1971). The UTM-proteins were localized in serial sections (7 μm) of endometrium by using a modification of the avidin-biotin immunoperoxidase staining procedure of Hsu et al. (1981). The primary antiserum, chicken antiserum to conjugated UTM-protein, was diluted 1:5,000–1:10,000 with 1% (v/v) normal goat serum in PBS. Controls consisted of preimmune chicken serum or antiserum preadsorbed with UTM-proteins, thyroglobulin, or the uterine milk protein-thyroglobulin conjugate. Second antibody (rabbit anti-chicken IgG) was used in excess at a dilution of 1:20 in PBS-1% normal goat serum. Peroxidase substrate, 3-amino-9-ethylcarbozole, was found to be optimal at 20 μg/ml. General staining conditions were those described by Vector Laboratories. Stained sections were examined under a Nikon Diaphot-TMD inverted microscope.

RESULTS

Protein Recovery from the Ligated Uterine Horn

Surgical restriction of the conceptus to one uterine horn had no apparent effect on maintenance of pregnancy or fetal development. That is, length of gestation was normal (144–146 days) and lambs were of normal weight. Uterine fluid was present in the ligated uterine horn of all pregnant animals, with the exception of one ewe at Day 30. Fluids were usually opalescent when freshly collected, but formed viscous clots upon standing at room temperature. Similar precipitates or clots were observed within the ligated uterine horns of several ewes after Day 90 of pregnancy.

Electrophoretic Analysis of Proteins in Uterine Fluids

Results from one-dimensional SDS-PAGE analysis of uterine fluid derived from three different ewes at Day 140 of pregnancy are shown in Figure 1. These gel patterns were fairly typical of all samples collected between Days 30 and 144 of gestation. The UTM-proteins appeared as two bands having apparent molecular weights of 57,000 ± 307 and 54,800 ± 210 ($\bar{X}$ ± SEM, n=9), with the upper band always predominating. Both polypeptides contained carbohydrate as indicated by positive staining with the periodic acid-Schiff procedure (results not shown). In one sample (Fig. 1, lane 3), two additional protein bands (M_r about 49,000 and 47,000) were also present in significant amounts. These two bands were detected in uterine fluid of several ewes, but usually

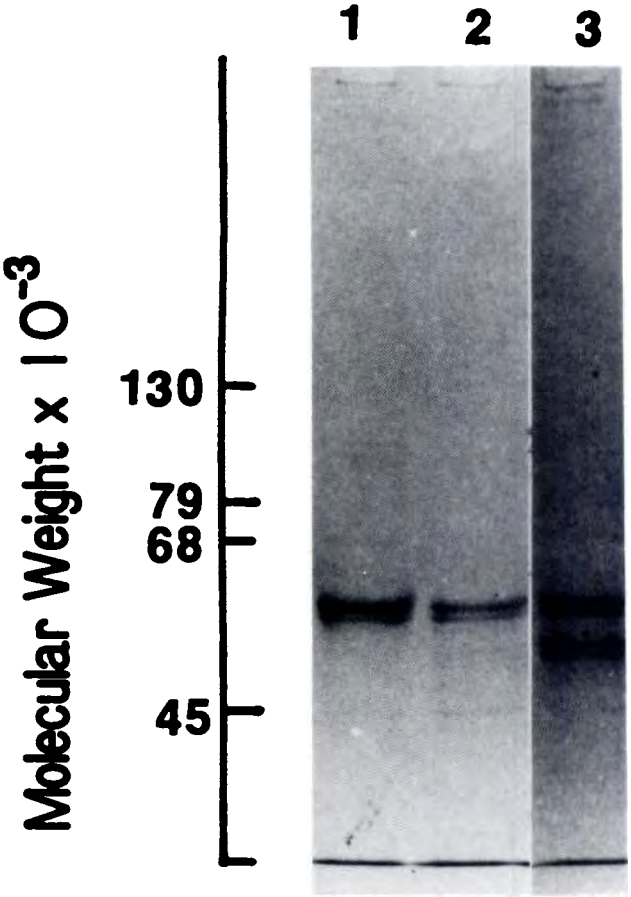

FIG. 1. One-dimensional polyacrylamide gel electrophoresis of uterine fluid from three different unilaterally pregnant ewes. Samples of uterine fluid from ewes at Day 140 of pregnancy were run in 5% (w/v) polyacrylamide gels. The amount of protein loaded in each lane was 10 μg as determined by the method of Bradford (1976). Gels were stained with Coomassie Blue.

in lower quantities than seen here. When the samples were stored unfrozen or were freeze-thawed repeatedly, these polypeptides and a series of pairs of components of even lower molecular weight began to accumulate (results not shown). It seems likely, therefore, that the smaller peptides resulted from partial proteolytic breakdown of the two UTM-proteins.

Analysis of uterine fluids by two-dimensional PAGE, in which isoelectric focusing was employed for separation in the first dimension, revealed the presence of traces of plasma proteins, including albumin, transferrin, and light and heavy chains of Ig at all stages of pregnancy (results not shown). However, the UTM-proteins were not resolved by the Ph 4.5–8.5 gradient used for isoelectric focusing. Analysis by NEPHGE demonstrated that the UTM-proteins were basic polypeptides that migrated at identical rates towards the cathode in the first dimension (Figs. 2c and d). They were evident as two bands (M_r = 57,000 and 55,000, respectively) and accounted for an increasing proportion of the total protein content of uterine secretions as pregnancy advanced. As noted with one-dimensional electrophoresis, other lower molecular weight proteins were often evident, particularly on gels overloaded with protein, e.g., as shown in Figure 2.

While the UTM-proteins were the dominant proteins of uterine fluid during pregnancy, they were not present in uterine flushings of nonpregnant ewes. The protein patterns in these ewes closely resembled those for blood plasma (Fig. 2a and b).

Purification of the UTM-Proteins

Sequential fractionation of uterine secretions from a ewe at Day 136 of pregnancy by CM-cellulose ion-exchange chromatography and gel filtration was used to isolate the two UTM-proteins from uterine fluids. Three peaks of basic protein were eluted from CM-cellulose columns by NaCl gradients (Fig. 3). Gel electrophoretic analysis of the two most basic protein peaks showed that the UTM-proteins were present in peaks 2 and 3, with the relative purity of the UTM-proteins being greater in peak 3 than in peak 2 (results not shown).

Subsequent gel filtration of peak 3 material on Sephacryl S-200 resolved a major symmetrical peak of apparent M_r of 55,000 plus minor amounts of protein of higher and lower molecular weight (Fig. 4). Material within the main peak also gave a positive reaction for neutral sugars by the phenol-sulfuric acid procedure. Analysis of this peak by electrophoresis revealed that it was composed almost entirely of the UTM-proteins. Heavily loaded gels, however, often revealed the 47,000–49,000 M_r proteins in trace amounts (< 5%).

Other purifications of UTM-proteins were done by using uterine fluid from each stage of gestation except Day 30. The purification scheme was identical except that basic proteins were eluted from CM-cellulose by elution with 0.5 M NaCl rather than by a salt gradient. The patterns of proteins resolved by Sephacryl S-200 were similar to that shown in Figure 4, and UTM-proteins in the major peak were similarly pure. Large-scale purification using batch-mode CM-cellulose chromatography followed by gel filtration using Sephadex G-100 was also a successful procedure for purifying the UTM-proteins.

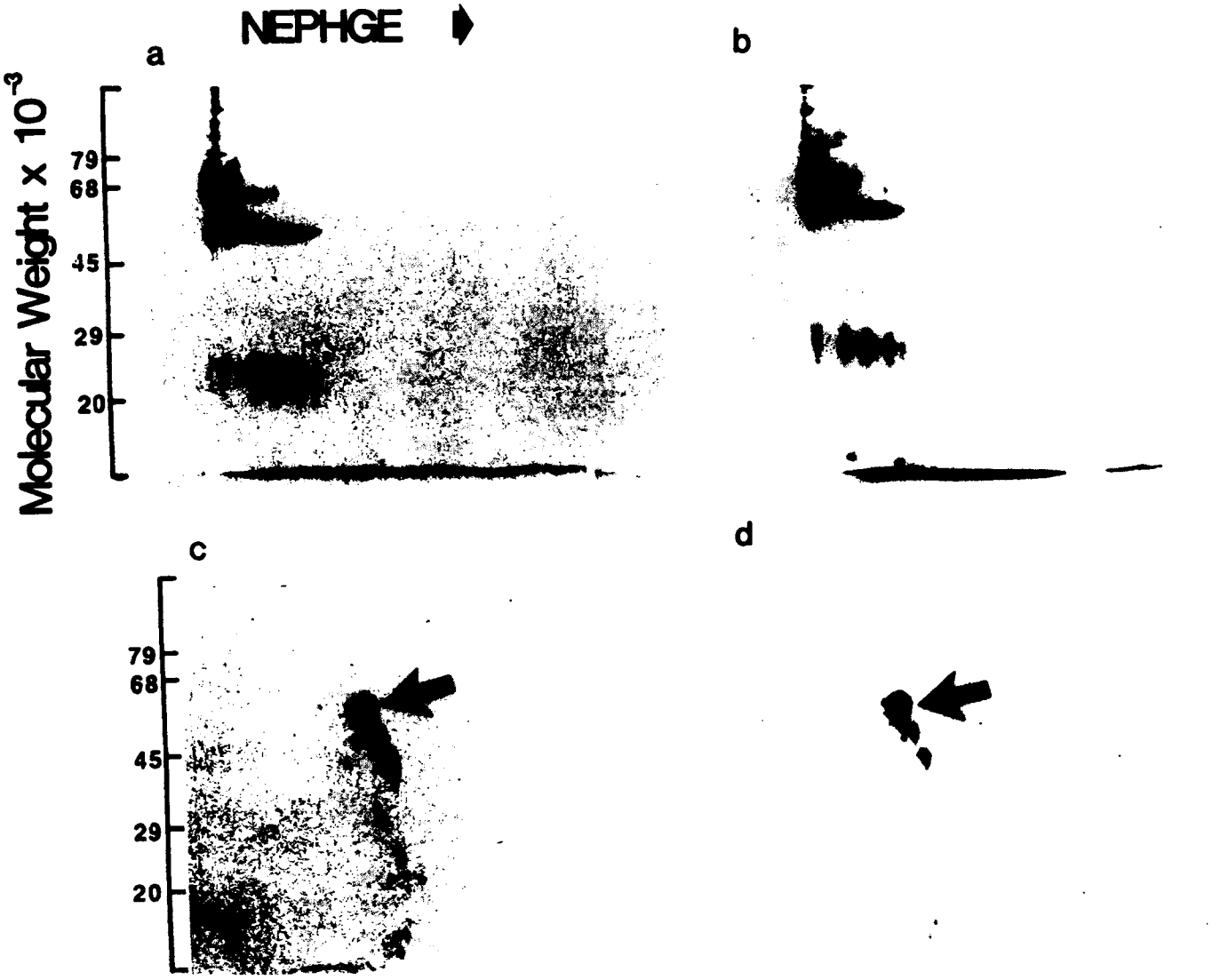

FIG. 2. Two-dimensional polyacrylamide gel electrophoresis of the basic proteins present in plasma and in uterine secretions. Samples were (*a*) plasma of a pregnant ewe at Day 140 of pregnancy, (*b*) uterine flushings of a nonpregnant ewe at Day 14 of the cycle, (*c*) uterine fluid of pregnant ewes at Day 120, and (*d*) Day 140 of pregnancy. Proteins were separated in the first dimension by NEPHGE and in the second dimension by SDS-PAGE in 10% (w/v) polyacrylamide gels. Proteins were stained with Coomassie Blue. Identical amounts of protein (200 μg) were loaded on each gel. Note that the predominant polypeptides (*arrows*) in the uterine secretions were a pair with M_r of 55,000–57,000. These polypeptides, called the uterine milk proteins, were absent in uterine flushings from nonpregnant ewes, which had a protein pattern similar to blood plasma.

Analysis of Proteins in Uterine Clots

The thick, viscous clots present in uterine milk were isolated by centrifugation at 10,000 × *g* for 10 min. This material was dissolved in 0.5 M dithiothreitol or in 0.1 M Tris-HCl buffer containing 20 mM dithiothreitol and either 6.8 M guanidine HCl or 6.0 M urea. One-dimensional PAGE of the dissolved material revealed that these clots were composed predominantly of UTM-proteins (results not shown).

Molecular Weight Estimation by Equilibrium Sedimentation

Weight-average molecular weight estimates of the purified UTM-proteins (from 0.6 to 1.5 mg/ml in 10 mM Tris-HCl buffer) averaged 50,680 (standard deviation = 4190). Estimates were also made on the proteins solubilized from the clots by means of 6 M guanidine-HCl containing 20 mM dithiothreitol. Analyses were carried out at three different con-

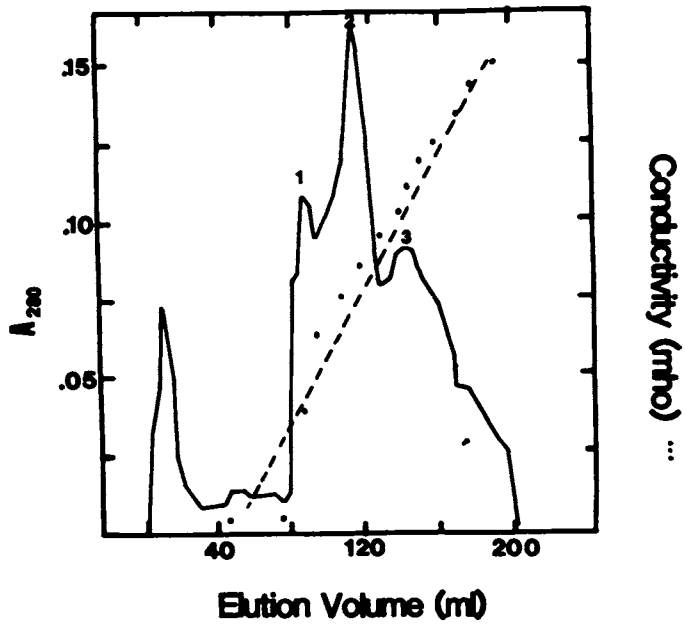

FIG. 3. Ion-exchange chromatography of uterine secretions from a ewe at Day 136 of pregnancy on a column of CM-cellulose. Proteins were eluted with a linear salt gradient of 0–0.5 M NaCl (———). The gradient was determined by measurement of salt conductivity (*mho*) (. .). The first peak represents acidic proteins not binding to the column, while peaks 1, 2, and 3 represent basic proteins. The UTM-proteins were present predominantly in peak 3. Protein was monitored by measuring absorption at 280 nm (A_{280}).

centrations of protein (0.6, 1.05, and 1.5 mg/ml), and weight-average molecular weight estimates averaged 51,465 (standard deviation = 1220). The Ln C versus r^2 plots were linear except for a small amount of curvature near the base of the cell.

Synthesis of UTM-Proteins by Cultured Endometrium

The rate of incorporation of L-[^{35}S] methionine (50 μCi) and L-[^{3}H] leucine (10 μCi) into non-dialysable secretory products was monitored throughout 24-h dual-label cultures of endometria from the ligated and pregnant uterine horns of a ewe at Day 140 of pregnancy. Patterns of incorporation of radiolabel over time were similar regardless of source of endometrium or radiolabel. Except for an initial lag period for cultures from the ligated uterine horn, there was a linear increase in accumulated protein until 24 h (Fig. 5).

Endometrial explants from the ligated and pregnant uterine horns of ewes at Days 30, 60, 90, 120, and 140 of pregnancy were cultured in the presence of L-[^{3}H] leucine for 24 h. As evaluated by two-dimensional PAGE, the major radiolabeled proteins present in culture medium from both the ligated and pregnant

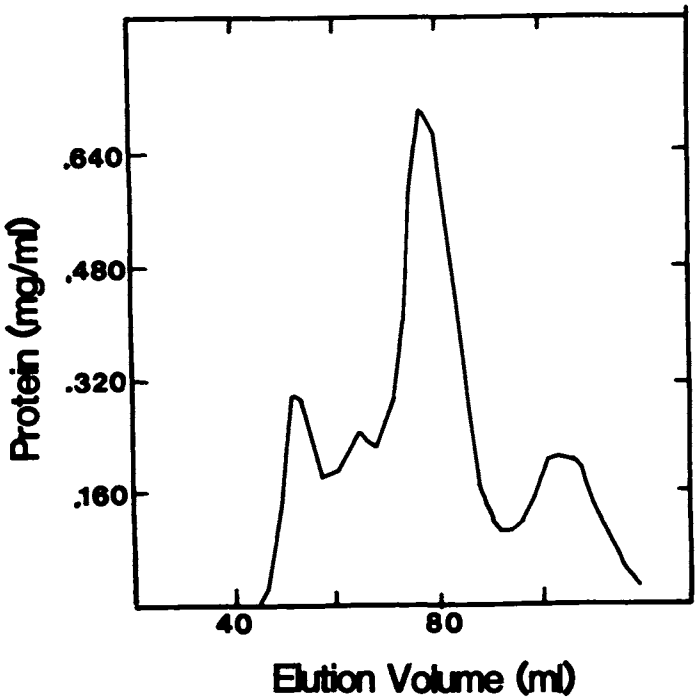

FIG. 4. Sephacryl S-200 gel filtration of partially purified UTM-proteins from Peak 3 material after CM-cellulose ion-exchange chromatography (see *Fig. 3*). Protein was measured colorimetrically (Lowry et al., 1951). Fraction size was 2 ml.

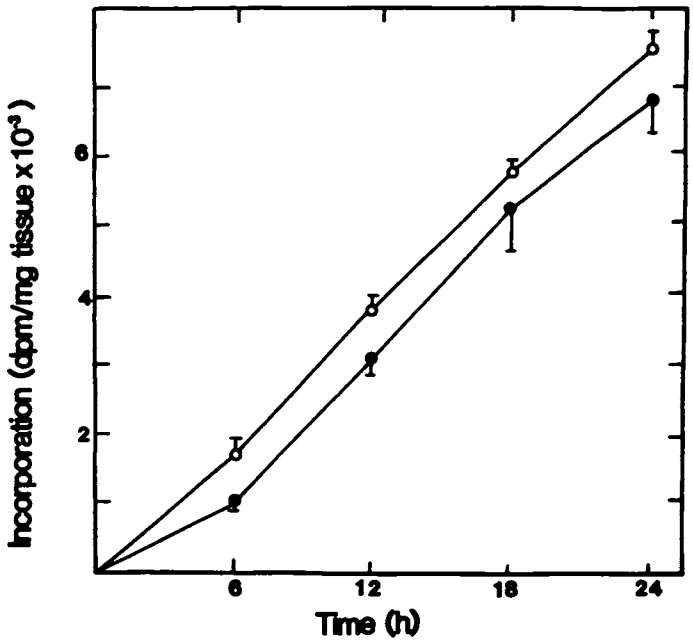

FIG. 5. Incorporation over time of L-[^{3}H] leucine into macromolecular components released into the culture medium by endometrial explants of pregnant (–o–o–) and ligated (–•–•–) uterine horns. Tissue (Day 140 endometrium) was cultured in triplicate in presence of 10 μCi of L-[^{3}H] leucine, as described in *Materials and Methods*.

uterine horns at all stages of gestation were the 55,000–57,000 M_r UTM-proteins. Representative fluorographs of proteins secreted by Day 60 and Day 90 pregnant endometrium are presented in Figure 6 (top row). Endometrium from nonpregnant ewes administered progesterone plus estrone for 120 days also released the two UTM-proteins into the culture medium (Fig. 6, third row). This suggested that production of the proteins was not dependent upon the presence of a conceptus in the uterus and that the UTM-proteins were steroid-induced. When compared to radiolabeled proteins present in endometrial tissue (Fig. 6, fourth row), it is apparent that the UTM-proteins were released into the medium selectively, i.e. they were probably secreted. The UTM-proteins were detectable in extracts prepared from endometrial tissue, but many other polypeptides were also present (Fig. 6, fourth row). Endometria from nonpregnant ewes and pregnant ewes prior to Day 20 of pregnancy did not produce detectable quantities of the UTM-proteins (results not shown).

Hormonal Control of UTM-Protein Production

Initial experiments indicated that endometrial tissue from ewes treated for a prolonged period (120 days) with a combination of progesterone plus estrone produce the UTM-proteins (Fig. 6). To confirm that UTM-proteins were synthesized in response to steroids, ovariectomized ewes were treated for 30 days with estrone, progesterone, a combination of both steroids, or with corn oil. Uterine secretions were recovered by aspiration of accumulated uterine fluid or by flushing the uterus with saline.

The UTM-proteins were not present in uterine secretions of ewes that had received corn oil (Fig. 7d) or estrone alone (Fig. 7a). The only detectable proteins in these flushings appeared identical to proteins present in serum collected from the same ewes (results not shown). The UTM-proteins were, however, major components of uterine flushings of all ewes treated with progesterone (Fig. 7b) or progesterone plus estrone (Fig. 7c). Endometrial explants derived from these ewes were cultured in the presence of L-[^{3}H]leucine for 24 h. The UTM-proteins were secreted only by explants from those ewes that had received progesterone or progesterone plus estrone (data not shown).

Immunocytochemical Localization of the UTM-Proteins

The distribution of the UTM-proteins within intercaruncular endometrial tissue was determined by immunocytochemical techniques. Antigen was detected in tissue sections obtained from ligated and

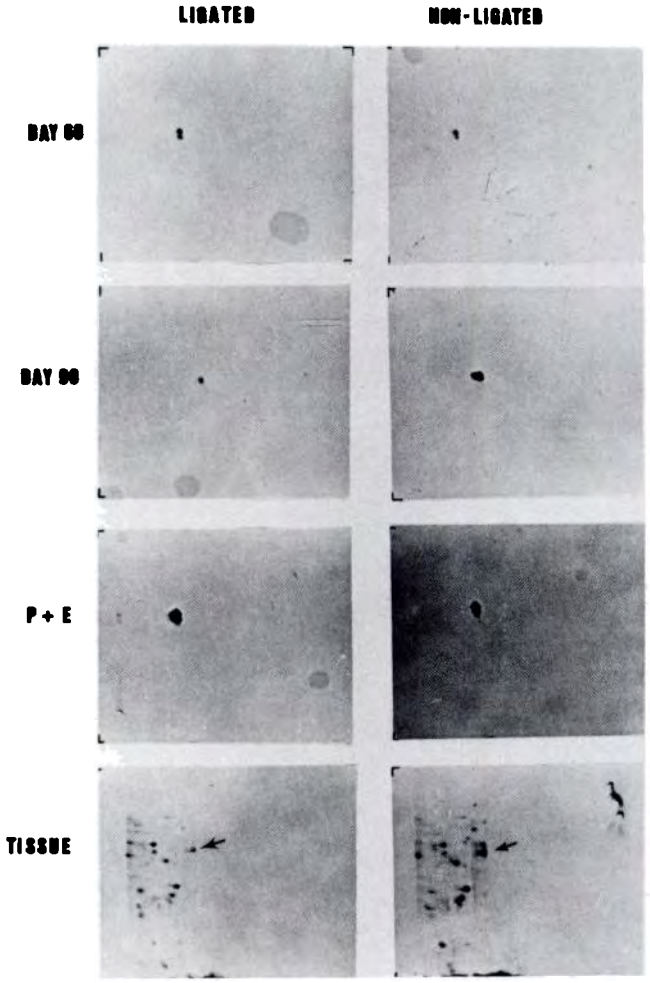

FIG. 6. Two-dimensional polyacrylamide-gel electrophoresis of proteins released into the medium by cultured explants of uterine endometrium. Samples of medium (about 0.5 ml of freeze-dried, dialyzed solution) from explants cultured in presence of L-[^{3}H]leucine (50 μCi) were separated in the first dimension by NEPHGE and in the second dimension by SDS-PAGE. Gels were dried and radioactivity was detected by fluorography. Gels on the *left* represent cultures established from the ligated horn; those on the *right* were from the nonligated horn. Represented are analyses of proteins secreted by endometrial explants from Days 60 and 90 of pregnancy and from a nonpregnant ewe treated with estrone and progesterone for 120 days. The *last row* shows the separation of radiolabeled proteins present in endometrial tissues from the ewe treated with steroids for 120 days. The locations of the UTM-proteins in the *lower two panels* are marked by *arrows*. Note that the endometrium from the nonligated horn produced similar products to that from the ligated uterine horn.

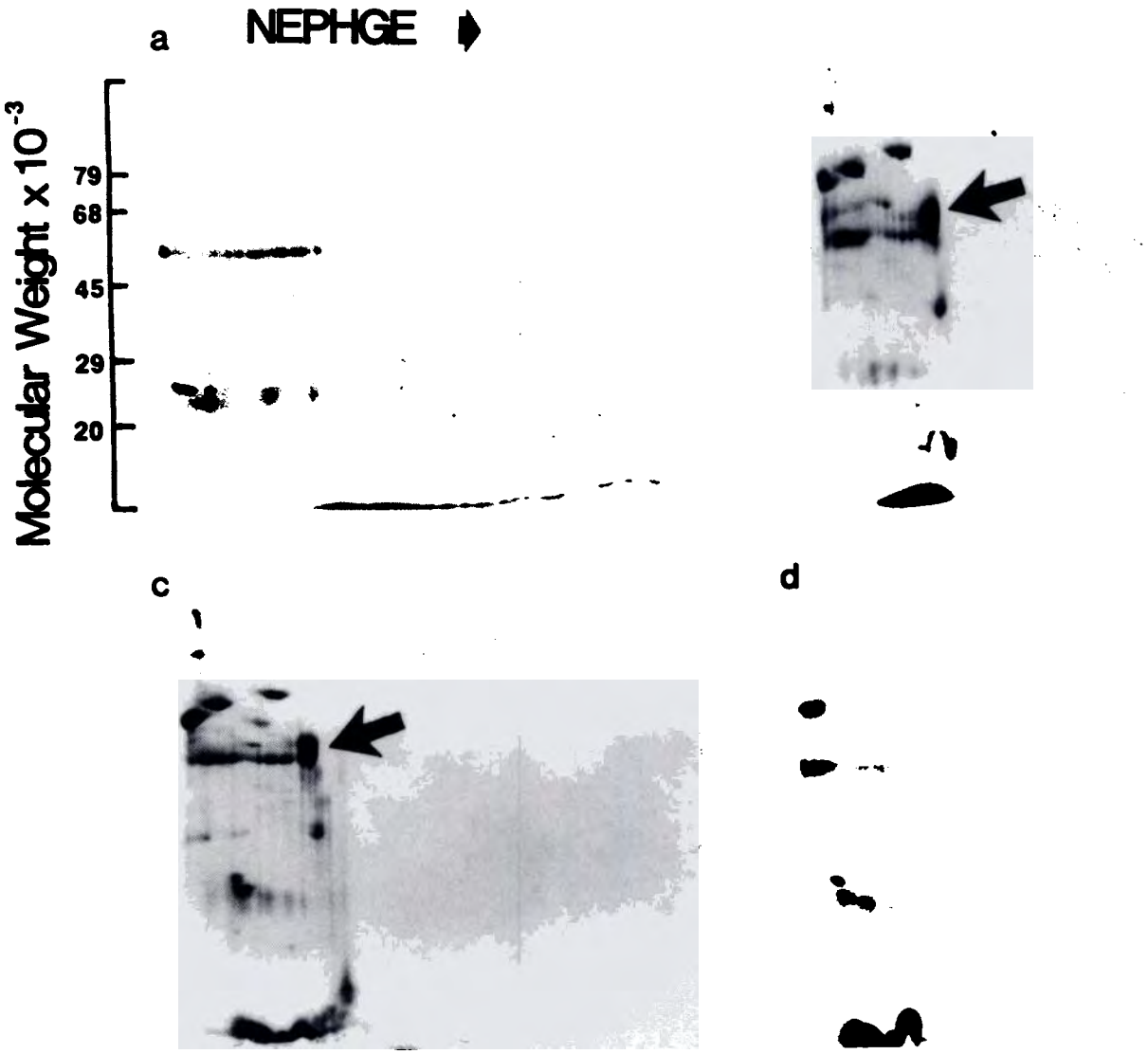

FIG. 7. Two-dimensional polyacrylamide gel electrophoresis of uterine fluids from ewes treated with steroids for 30 days. Proteins were separated by NEPHGE and SDS-PAGE in 10% (w/v) polyacrylamide gels and stained by Coomassie Blue. The *four panels* represent secretions of ewes treated with (*a*) estrone, (*b*) progesterone, (*c*) progesterone plus estrone, or (*d*) corn oil. Note that the UTM-proteins (*arrows*) were found only in uterine fluids of ewes treated with progesterone or progesterone plus estrone. Approximately 100–200 μg of protein were loaded onto each gel.

pregnant uterine horns of ewes at all times examined (Days 60, 90, 120, and 140 of gestation). As shown in Figure 8 for endometrium at Days 60 and 120, specific staining for UTM-proteins was localized primarily along the apical borders of both glandular and luminal epithelial cells. It is also clear, however, that UTM-proteins were associated with the basal and lateral borders of the cells. Specific staining was observed in secretory material and debris in the lumina of uterine glands. No specific staining was found in stromal or myometrial tissue layers (data not shown for the latter). No staining was observed in sections of endometrium collected during the estrous cycle or in sections of kidney, small intestine, liver, thymus, or thyroid from a fetus at Day 140 of gestation.

DISCUSSION

Results of these studies demonstrate that a major portion of the proteins in uterine secretions produced by pregnant ewe is comprised of a pair of basic glycoproteins with similar molecular weights and isoelectric points. We have named these glycoproteins the UTM-proteins because of their association with the milky-appearing secretions of the uterus. These

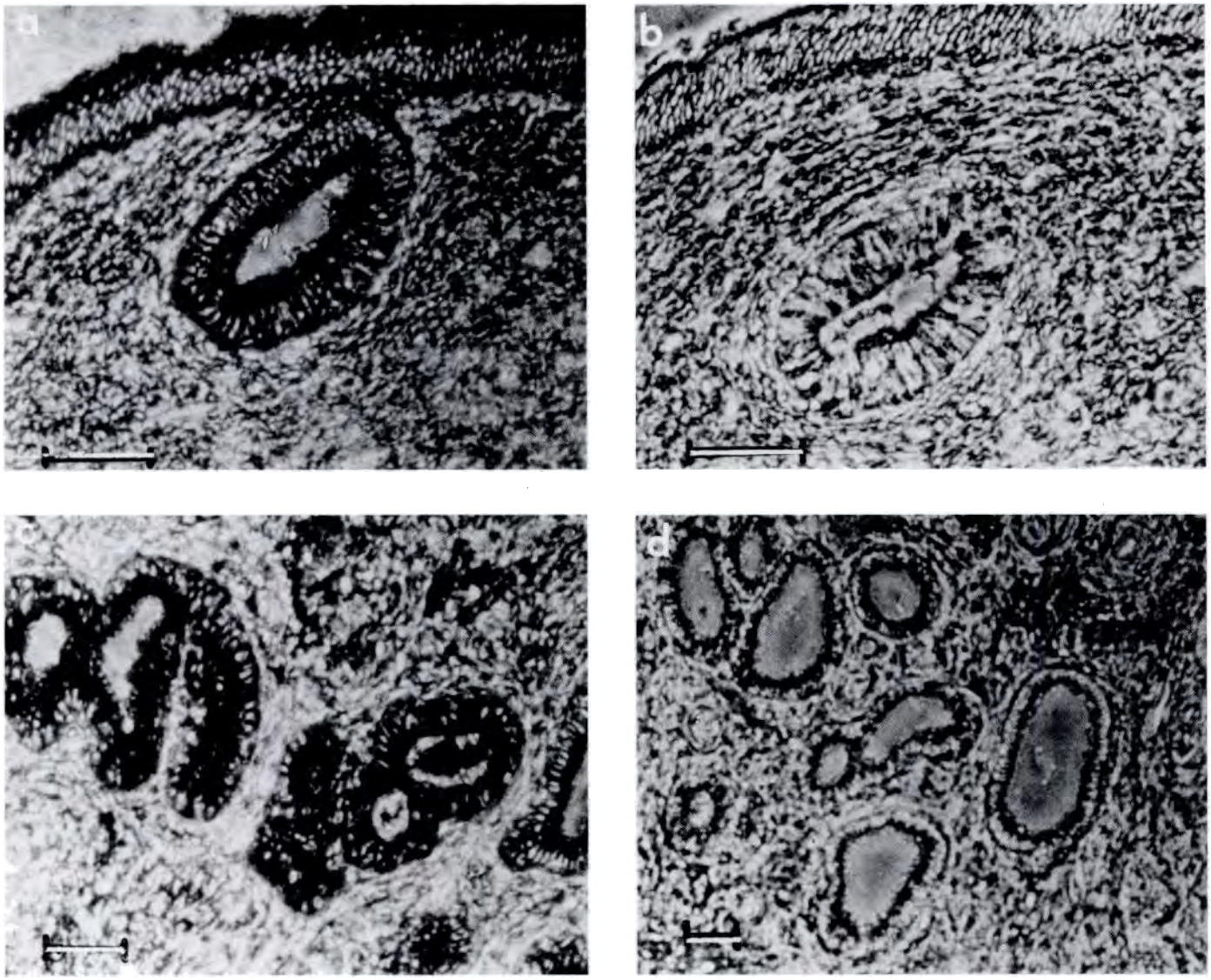

FIG. 8. Immunocytochemical localization of UTM-proteins by the avidin-biotin immunoperoxidase-staining procedure in endometria from pregnant ewes. *Panels* (*a*) and (*b*) show secretions from the ligated uterine horn of a unilaterally pregnant ewe on Day 120. Section (*a*) was treated with immune rooster serum (diluted 1:7500 with PBS) while the closely adjacent section in (*b*) was treated with immune-adsorbed antiserum at a similar dilution. Both sections were counterstained by hematoxylin. In (*a*), note specific staining for UTM-proteins associated with the surface and glandular epithelium. *Panel* (*c*) is an endometrial section showing specific staining in the deeper uterine glands at Day 120 (antiserum dilution 1:7500). *Panel* (*d*) is a section of endometrium from the pregnant uterine horn of a Day 60, unilaterally pregnant ewe (antiserum dilution 1:7500). The *bar* in each panel represents 50 μm.

two glycoproteins are virtually the only two proteinaceous components that can be resolved by two-dimensional PAGE of uterine secretions obtained between Day 30 and 144 of pregnancy. Only traces of serum proteins are present in the uterine fluids, and the major contaminants were basic polypeptides that probably represent proteolytic breakdown products of UTM-proteins.

Two lines of evidence indicate that the UTM-proteins are secretory products of the endometrium. First, radiolabeled UTM-proteins were major polypeptide components recovered from medium in which endometrial explants were cultured. That their presence was the result of secretion and not of tissue breakdown was suggested by the fact that they were only minor components of tissue extracts, and that most radioactive tissue proteins were not detected in culture medium. Immunocytochemical localization of UTM-proteins in luminal and glandular epithelium of intercaruncular endometrium, the presumed sites of

uterine secretory activity, provides a second line of support for the UTM-proteins being secretory products. Their presence within the glandular lumen and localized concentration in the apical regions of cells is also consistent with this view. However, the reason for their apparent association with the basal and lateral edges of the cells (Fig. 8a) is not clear.

By restricting the conceptus to one uterine horn and then collecting uterine secretions from the nongravid horn of near-term ewes, it was possible to obtain a volume of fluid that contained an average 15 g of total protein (R. J. Moffatt, F. W. Bazer, and R. M. Roberts, unpublished observations). In addition, the uterine milk often contained large proteinaceous clots composed largely of UTM-proteins. This clot material provides another potential source of the proteins. The abundance of these materials and the relative ease by which they can be purified allows very large quantities of these unusual proteins to be prepared. Ligation of the uterus does not appear to alter the pattern of proteins produced by the endometrium since the type of proteins secreted by endometrium in vitro was similar for ligated and nonligated horns.

In nonpregnant ewes, UTM-proteins were synthesized and secreted only in response to progesterone or progesterone plus estrone. Estrone alone had no effect. The proteins were not present during the luteal phase or in ovariectomized ewes treated with progesterone for only 15 days (R. J. Moffatt, F. W. Bazer, and R. M. Roberts, unpublished observations), which suggests that induction of synthesis and secretion of the proteins requires long-term exposure to progesterone. We know of no other mammalian system where a group of progesterone-induced proteins are secreted in such abuundance. Uteroglobin, the progesterone-dependent protein of the rabbit uterus, is produced transiently during ealy pregnancy or pseudopregnancy (Bullock and Connell, 1973; Beier, 1974; Kopu et al., 1979), but only a few milligrams can be purified from such animals. The pig secretes relatively large amounts of a variety of proteins in response to progesterone. However, the most abundant of these, uteroferrin, rarely accumulates in amounts exceeding 0.5 g/animal (Roberts and Bazer, 1980). The sheep, therefore, should provide an excellent model for studying the progestational control of gene expression and synthesis of what appears to be a particularly abundant uterine product of a higher mammal.

The function of the UTM-proteins is unclear. They do not bind calcium or prostaglandin $F_{2\alpha}$ (R. J. Moffatt and F. W. Bazer, unpublished observations), two substances found in abundance in uterine fluid of the unilaterally pregnant ewe (Harrison et al., 1976; Bazer et al., 1979). Because induction of their secretion requires prolonged exposure to progesterone, they probably are not involved in events in early pregnancy. Nonetheless, that they are produced in such large amounts and appear only during pregnancy suggests that they serve an important function during gestation.

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