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Placental Transport and Distribution of Uteroferrin in the Fetal Pig

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ABSTRACT

Placental transport of uteroferrin (Uf), the progesterone-induced iron transport glycoprotein, and its distribution within the fetus were investigated by the peroxidase-antiperoxidase bridge (PAP) technique. In Experiment 1, Uf was localized in endometrial and placental tissues taken from gilts on Days 60, 75, 90 and 105 of pregnancy. Uteroferrin was observed within cells of the endometrial glands and surface epithelium adjacent to placental areolae but not in endometrial surface epithelium between areolae. Heavy staining for Uf was observed in cells of the areolae and was associated with both supra- and infranuclear cytoplasmic vesicles. Vesicles located within the infranuclear cytoplasm were occasionally observed to be releasing their contents into capillaries surrounding the areolae. In Experiment 2, Uf was measured by radioimmunoassay in blood samples taken from the umbilical vein and artery of fetuses on Day 75 of pregnancy. Uteroferrin concentrations were greater ($P<0.07$) in umbilical vein blood (79.8 ± 13.1 ng/ml) than in umbilical artery blood (43.9 ± 13.1 ng/ml). Uteroferrin binding by Day 75 fetal liver membranes was examined in Experiment 3. Binding of ^{125}I-Uf increased linearly with increasing quantities of membrane protein and binding of ^{125}I-Uf was competitively inhibited by adding unlabeled Uf to the assay. In Experiment 4, urine samples were taken from Day 75 fetuses and assayed for β-mercaptoethanol activated acid phosphatase activity which is indicative of Uf. In addition, urine proteins were analyzed for Uf by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and Ouchterlony double immunodiffusion (ID). Acid phosphatase specific activity was 3.37 ± 1.83 µmol substrate hydrolyzed/10 min per mg protein. Uteroferrin was detected in two of three urine samples by 2D-PAGE and in three of eight samples by ID. In Experiment 5, tissue distribution of Uf was determined by the PAP technique in liver and kidney tissue taken from fetuses on Day 75 of pregnancy. Staining for Uf was observed in collecting ducts and proximal tubules of kidney tissue, but staining was not observed in liver tissue.

These results indicate that Uf is transported by the areolae into the chorioallantoic capillaries and to the fetus by the umbilical vein. Within the fetus Uf is either bound by the liver, probably to supply iron for hematopoesis, or cleared by the kidney and transported within the urine to the allantoic sac to serve as a temporary iron storage reservoir.

INTRODUCTION

Establishment of an epitheliochorial placenta in the pig (Grosser, 1909) and a number of other animals including the horse, camel and whale (Amoroso, 1952) causes little or no destruction of either the uterine mucosa on the maternal side or chorionic epithelium on the fetal side. Final attachment of the conceptus is achieved by interdigitation of epithelial microvilli and not by invasive growth (Brambel, 1933). The blood supply of mother and fetus are therefore separated by several tissue layers and macromolecules required for embryonic growth and development are released in the form of secretion by the glandular endometrium of the uterus. These secretions are absorbed by the chorion at specialized cup-shaped regions called areolae which form opposite the uterine glands (Fig. 1; Amoroso, 1952; Chen et al., 1975). The absorptive function of the chorionic areolae was recently examined using transmission electron microscopy by Friess et al. (1981). These authors observed numerous coated vesicles and tubules immediately beneath the microvilli in the apical cytoplasm of the areolar epithelium. The content of most of these vesicles appeared similar in electron

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density to substances within the areolar lumen, supporting the suggestion that secretions were being actively absorbed for use by the conceptus.

In a recent review, Roberts and Bazer (1980) discussed uteroferrin (Uf), a progesterone-induced basic glycoprotein secreted by the uterine glandular epithelium of the pig, which contains one molecule of iron (Fe) and possesses an acid phosphatase activity that is enhanced by reducing agents (Murray et al., 1972, Knight et al., 1973; Schlosnagle et al., 1974; Buhi et al., 1982b). Uteroferrin is produced in large amounts, particularly in midpregnancy when synthesis may exceed 1 g/day (Basha et al., 1979), and is believed to function in Fe transport from mother to fetal-placental unit (Roberts and Bazer, 1980; Buhi et al., 1982a; Ducsay et al., 1982).

Since allantoic fluid of midpregnant gilts also contains Uf (Bazer et al., 1975) of maternal origin (Ducsay et al., 1982) it must be transported intact across the chorioallantois (placenta). It has been suggested that Uf-mediated Fe transport to the fetus is by direct passage across the chorioallantois into allantoic fluid, from where Fe is distributed to the fetus. In support of this, Buhi et al. (1982a) demonstrated that the Fe on Uf in allantoic fluid was rapidly transferred to transferrin which could gain access to the fetal circulation and transport Fe to centers of hematopoesis, particularly the liver (Ducsay et al., 1982). However, other data indicated that, although Fe in the allantoic sac is in a dynamic state, accumulation of Fe within allantoic fluid does not precede transfer of Fe to fetal tissues (Ducsay, 1980). This study was designed to critically examine Uf transport across the placenta and determine its subsequent distribution with the fetal-placental unit.

MATERIALS AND METHODS

Animals and Surgical Procedures

Gilts with two previous estrous cycles of normal duration (18–22 days) were checked daily for estrus with intact boars and bred when estrus was detected and at 12 and 24 h after detection of estrus. Surgical procedures were performed with gilts under general anesthesia and tissues were obtained after exposure of the reproductive tract by midventral laparotomy (Knight et al., 1977).

Experiment 1

Uterine endometrium with apposed chorioallantois was obtained from gilts on Days 60, 75, 90 and 105 of pregnancy (n=4, one gilt/day) to localize Uf by immunohistochemical staining. Tissues were cut to 3–5 mm^3 pieces and fixed overnight at 4°C by immersion in a solution of freshly prepared 2% (w/v) paraformaldehyde, 0.1% (w/v) glutaraldehyde in 0.05 M sodium phosphate buffer, pH 7.0. Fixed tissues were washed, dehydrated, embedded in Paraplast and sectioned at 7 μm.

Uteroferrin was localized in tissue sections by the peroxidase-antiperoxidase (PAP) bridge technique (Moriarty et al., 1973) utilizing the double bridge modification reported by Ordronneau (1979). Antiserum to Uf was that of Chen et al. (1973) and was used at a dilution of 1:10,000 in phosphate buffered saline (PBS) containing 1% (v/v) normal rabbit serum (PBS/NRS). Freshly deparaffinized tissue sections were rinsed in PBS for 3 min and subsequently incubated with 2% (v/v) PBS/NRS for 3 min to reduce nonspecific staining. Treatment with normal rabbit serum was repeated prior to adding each PAP component. Solutions were added dropwise to horizontally placed slides. After the first PBS/NRS rinse sections were incubated with antiserum to Uf for 48 h at 4°C in a humidified chamber. To terminate the incubation, slides were washed three times by immersion in PBS (3 min each wash). The second PAP component, rabbit anti-sheep gamma globulin (Cappel Labs., Cochranville, PA), was diluted to 1:100 with PBS/NRS, incubated with the tissue for 10 min at room temperature and washed as before. The third PAP component, sheep peroxidase-antiperoxidase (Cappel Labs.) was added to sections at a dilution of 1:150 for 10 min at room temperature. The incubation was terminated by washing with PBS. To achieve formation of the double bridge (Ordronneau, 1979), rabbit anti-sheep gamma globulin and sheep peroxidase-antiperoxidase were reapplied stepwise to the tissue as previously described. To stain the tissue, a solution of 75 mg/100 ml of 3,3′ diaminobenzidine tetrahydrochloride (DAB; Sigma Chemical Co., St. Louis, MO) in 0.05 M, pH 7.6 Tris-HCl buffer was prepared (Nakane and Pierce, 1966), filtered (Whatman #1; Whatman Ltd., England) and hydrogen peroxide added to a final concentration of 0.002% (w/v; Petrusz et al., 1975). Slides were immersed in this solution for 8 min with constant slow stirring. The slides were then washed with 0.05 M Tris-HCl buffer (pH 7.6) for 2 min and finally in PBS for 2 min. Some sections were counterstained with Mayer's hematoxylin for 4 min.

The control experiments described below were conducted to determine the specificity of the PAP procedure (Petrusz et al., 1976). In the first experiment, components of the stain were sequentially replaced by PBS to ascertain the staining contribution of each component through direct binding to the tissue. The second experiment involved application of the staining procedure to porcine spleen, a tissue known to be devoid of Uf (Chen et al., 1973), to detect nonspecific staining. In the third control experiment, the primary antiserum was used at sequentially higher dilutions. Thus, staining of areas by undetermined antibodies can be identified. The fourth control involved absorption of the specific antisera with increasing amounts of purified Uf for 2 days at 4°C before PAP staining. As a final control, NRS was used instead of antibody to Uf. In addition to tissue control experiments, the primary antiserum was

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utilized in a radioimmunoassay (see Experiment 2) and did not cross-react with either transferrin or lactoferrin, two iron containing glycoproteins with some similarities to Uf (Roberts and Bazer, 1980) and found in biological fluids. Tissue sections were examined for staining and photographed using a Wild M20 compound microscope.

Experiment 2

Immunoreactive Uf was measured in fetal (n=17) umbilical arterial and venous plasma by a double antibody radioimmunoassay (RIA) procedure. These fetuses were from two gilts laparotomized on Day 75. The uterus was exposed to allow exteriorization of each fetus through an incision in the adjacent antimesometrial wall of the uterus, chorioallantois and amnion. Blood samples (5 ml) collected from an umbilical artery and the umbilical vein in heparinized syringes (100 IU/sample) were chilled and centrifuged at 7700 X *g* to obtain plasma which was stored at −20°C until assayed.

The RIA was performed with sheep antiserum to Uf described in Experiment 1. The assay buffer contained 20 mM barbital, 0.9% (w/v) NaCl, 0.029% (w/v) EDTA and 0.25% (w/v) bovine serum albumin (BSA) and was adjusted to pH 8.0 with 1.0 N hydrochloric acid. Tracer (^{125}I-Uf) was prepared by the Iodo-Gen technique (Markwell and Fox, 1978 Markwell, 1978) using the procedure of Buhi et al. (1982a). Uteroferrin was assayed in 0.1 ml samples of fetal plasma to which 0.1 ml assay buffer was added. Standards (0.1 ml) dissolved in assay buffer received 0.1 ml of hysterectomized gilt plasma. Buffer (0.4 ml) and the specific antiserum (0.1 ml; 1:10,000 dilution) were then added and the assay held at 4°C for 24 h. In a series of prior experiments it was established that this dilution of antiserum precipitated 30% of the tracer in 0.1 ml of ^{125}I-Uf. Following the addition of ^{125}I-Uf (0.1 ml; 20,000 cpm) the assay was incubated for an additional 24 h. Next, sheep γ-globulin (0.1 ml; 400 μg/ml; ICN Nutritional Biochemicals, Cleveland, OH) and rabbit antibody raised against sheep γ-globulin (0.1 ml; 1:3 dilution; Antibodies Inc., Davis, CA) were added, and the assay incubated 48 h. After incubation, tubes were centrifuged at 2250 X *g* for 20 min, washed with cold assay buffer, recentrifuged and the pellet counted in a γ-counter. The specific antiserum did not cross-react with transferrin (2 mg/ml) or lactoferrin (1 mg/ml). Addition of 2 and 50 ng of Uf yielded values ($\bar{X}$ ± SEM) of 2.0 ± 0.3 ng (n=7) and 50.3 ± 3.8 ng (n=4), respectively. The working range of the assay was from 1–2 ng to 50–60 ng and there was no evidence of nonparallelism between the standard curve and a curve produced by adding an increasing volume of a plasma sample containing purified uteroferrin ($Y=-3.03X_1 + 7.31$ VS $Y=-2.71X_2 + 4.31$ where X_1 =log of volume and X_2 =log of standard curve dose). The minimum detectable value that was different from 0 was determined to be 0.5 ng during validation of the assay. Intra- and inter-assay coefficients of variation were 10.4 and 7.1%, respectively.

Data were analyzed by least squares analysis of variance to determine effects of gilt, fetuses within gilt, source of sample (umbilical artery or vein) and gilt by source of sample interaction.

Experiment 3

In a preliminary study, Uf binding by fetal liver membranes was examined. Livers were removed from fetuses obtained by hysterectomy of gilts on Day 75 of pregnancy in Experiment 2. Crude liver plasma membrane was prepared by a modification of the method of Ray (1970). Tissues were minced in cold saline (0.9% [w/v] NaCl) and washed several times to remove blood. Samples of blotted tissue (approx. 5 g) were homogenized in a Dounce tissue homogenizer (0.64–1.40 mm clearance; Wheaton, Millville, NJ) with 40 ml of homogenization buffer (0.5 M $CaCl_2$ –1 mM $NaHCO_3$, pH 7.5) until most cells were broken, but nuclei remained intact. All homogenization and storage solutions contained 1 mM protease inhibitor (phenylmethylsulfonyl fluoride; Sigma Chemical Co.). The homogenate was diluted to 500 ml in homogenization buffer and held on ice for 5 min to allow lysing of unbroken cells. This solution was then filtered through cheesecloth and centrifuged at 1500 X *g* for 30 min (GSA rotor, Dupont Instruments-Sorvall, Newtown, CT). The supernatant fraction was aspirated taking care not to disturb the buffy membrane layer above the pellet. The total pellet was resuspended by homogenization, diluted to 250 ml and centrifuged at 1500 X *g* for 15 min. Aspiration, resuspension, dilution (150 ml) and centrifugation were repeated. After removal of the final supernatant, the buffy membrane layer was gently washed from the pellet and stored in sucrose (0.25 M) at −20°C.

For liver binding analyses, isolated membranes (in 0.25 M sucrose) were centrifuged at 27,100 X *g* (SS-34 rotor; Dupont instruments-Sorvall, Newtown, CT) for 20 min and the pellet retained for the assay and protein determination. To determine membrane protein, 0.5 ml of the isolated membranes in 0.25 M sucrose plus 1.5 ml of 1N NaOH were placed in a boiling water bath and shaken until clear. Protein was quantified in this solution by the method of Lowry et al. (1951) using BSA as the standard. A membrane stock solution was prepared by adding a volume of assay buffer (25 mM Tris-HCl, 10 mM $CaCl_2$, 0.1% BSA, pH 7.6) to the pellet to give 3 mg membrane protein/ml. In the Uf binding assay each tube contained ^{125}I-Uf (20,000 cpm) and liver membrane (75–600 μg protein) brought to a total volume of 0.5 ml with assay buffer (all tubes were duplicated). Competitive displacement of bound tracer was accomplished by adding radioinert Uf (2.5–400 μg). Assays were incubated for 90 min at 25°C in 1 ml polypropylene centrifuge tubes. After incubation, tubes were centrifuged (Eppendorf Microfuge, Brinkmann Instrument, Inc., Westbury, NY) for 4 min. The pellet obtained was washed with buffer, recentrifuged and the final pellet counted.

Experiment 4

Urine samples were collected from Day 75 fetuses (n=27) obtained at laparotomy from gilts in Experiments 2 and 5. Fetuses were exteriorized as previously described, umbilical cords tied and severed, and the abdominal wall opened to expose the bladder. Urine samples were taken with a 5-ml syringe fitted with a 22-gauge needle and stored at −20°C until analysis. Protein concentration was determined by the

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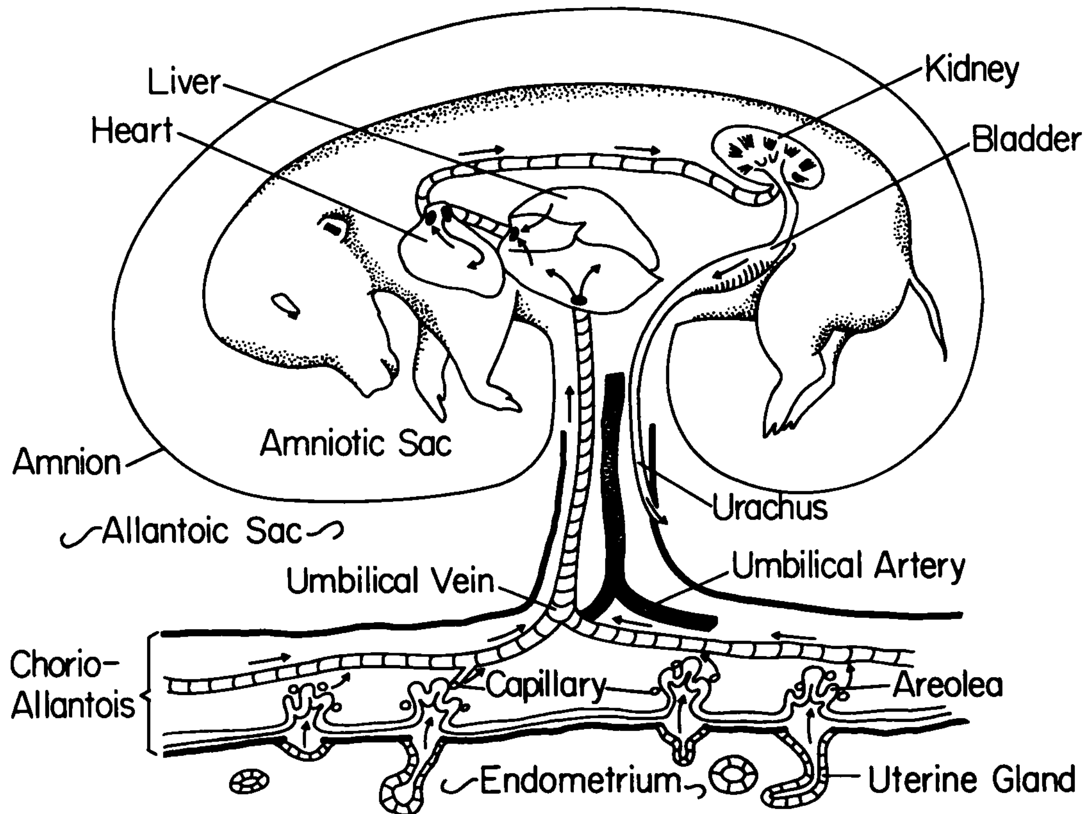

FIG. 1. Proposed route of Uf transport from the uterus to the fetolplacental unit. Uteroferrin secreted by the uterine glands is pinocytosed by cells of the areolae, released into the chorioallantoic capillaries and transported to the fetus via the umbilical vein. Within the fetus, Uf is bound by the liver, presumably to supply iron for hematopoesis. Uteroferrin not removed by the liver is partially cleared by the kidney, accumulates within the bladder, and enters the allantoic sac in urine released through the urachus.

method of Lowry et al. (1951) using BSA as standard. Acid phosphatase activity was measured in sample aliquots treated with β-mercaptoethanol (Schlosnagle et al., 1974; Buhi et al., 1982b). Following acid phosphatase analysis, samples were randomly assigned to agar gel double immunodiffusion analysis (n=8) or polyacrylamide gel electrophoresis (n=3). This was necessary as small sample volumes and low protein content prevented analysis by both techniques. For immunodiffusion (Ouchterlony, 1958), urine samples were concentrated by lyophilizing 1-ml aliquots and resuspending the sample in 0.1 ml of PBS. Urine concentrates (10 μl) or Uf standard were applied to wells in the agar (0.1% [w/v] Noble agar, DIFCO Laboratories, Detroit, MI) of immunodiffusion plates and developed against antiserum to Uf at room temperature in a humidified chamber. Plates were photographed without prior staining. For 2D-PAGE, urine sample aliquots containing 200 to 450 μg protein were lyophilized and the dried material dissolved in 150 μl of 5 mM K_2CO_3 containing 9.4 M urea, 2% (v/v) Nonidet P-40 and 0.5% (w/v) dithiothreitol (Horst and Roberts, 1979). Samples were electrofocused by the nonequilibrium pH gradient electrophoresis technique

FIG. 2. Uterine endometrium from a gilt on Day 60 of pregnancy stained for Uf by the peroxidase-antiperoxidase method. *A*) Staining for Uf was present in the apical cytoplasm of endometrial epithelial cells of the uterine glands (*G*) and within the lumena (*L*) of the glands. Staining was not present in maternal arteries (*MA*) or veins (*MV*) or within the endometrial stroma (*ST*). X575. *B*) Adjacent section to the section shown in *A*. The peroxidase-antiperoxidase method was performed with normal sheep serum in place of antiserum to Uf. Positive staining is not present. X575. *C*) Surface epithelium of the endometrium (*E*) contained staining for Uf (*arrows*) in cells adjacent to the chorioallantoic areolae (*AR*) but not in cells of the surface epithelium outside the areolae. Hematoxylin counter-stained. X1600.

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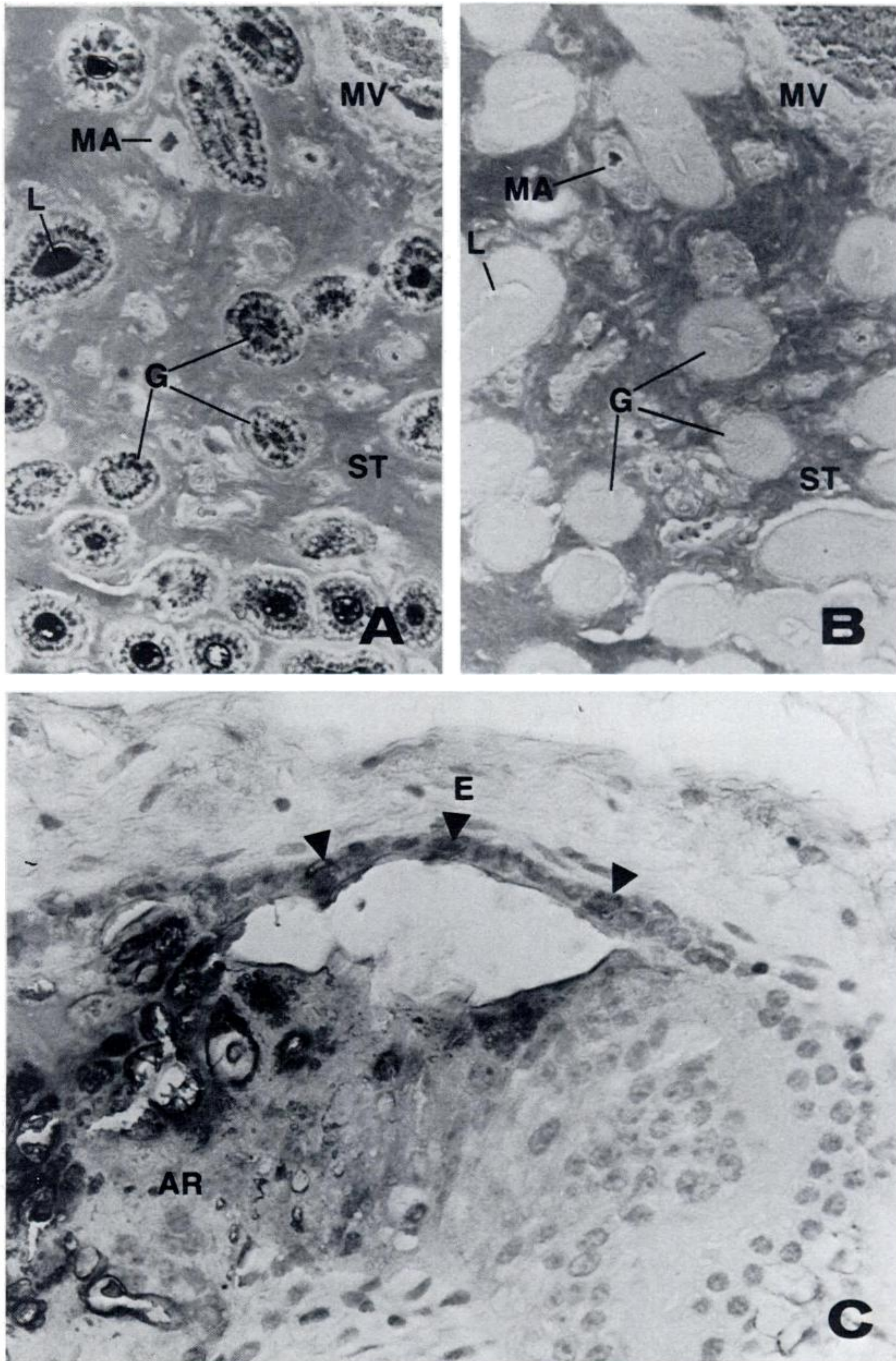

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(NEPHGE) for basic proteins as described by Basha et al. (1980). Protein migration was toward the cathode and the duration of electrofocusing was 3.5 h. Sodium dodecylsulfate-gel electrophoresis in the second dimension was performed by the methods of Basha et al. (1980) and Laemmli (1970). Slabs were fixed and stained with Coomassie blue R-250 before photography.

Experiment 5

Two pregnant gilts were hysterectomized on Day 75 and fetal liver and kidney tissue obtained to localize Uf by immunocytochemical staining. Liver tissue was fixed by immersion in the fixative as described in Experiment 1. Kidneys were perfused with fixative via the renal artery until the tissue blanched. Kidneys were subsequently bisected and fixation continued by immersion. Uteroferrin was localized by the PAP procedure described in Experiment 1.

RESULTS

Experiment 1

The relationship between the maternal uterine endometrium and fetal-placental unit is shown in Fig. 1. Positive Uf staining was observed in the lumen and epithelial cells of the uterine glands on Days 60, 75, 90 and 105 of pregnancy. In Fig. 2 endometrium from a Day 60 animal is shown and this staining pattern was present on all days examined. Within the glandular epithelium Uf appeared localized primarily in the apical cytoplasm above the large clear nucleus. The stromal cells and maternal blood vessels did not stain. Staining was present in endometrial surface epithelial cells adjacent to the chorionic areolae, but not in other cells of the surface epithelium (Fig. 2C).

When chorionic tissues were examined, cells of the areolae could be distinguished by their large size and content of vesicles. The latter stained intensely for Uf (Fig. 3A). Staining was concentrated in large and small vesicles located in both the supra- and infranuclear cytoplasm of the cell (Fig. 3A). In particular, there were high concentrations of infranuclear vesicles located in cells immediately adjacent to chorioallantoic capillaries (Fig. 3B). In some sections positive staining was seen at the border of capillaries and in some instances appeared to be in the process of being released from cells of the areolae into a capillary (Fig. 3C). Positive staining for Uf was not observed in the mesenchymal tissue of the chorioallantois, epithelial cells of the allantois or chorionic epithelial cells outside the areolae. The described staining pattern was present on all days examined and control experiments confirmed the specificity of staining for Uf.

Experiment 2

Immunoreactive Uf was detected in plasma from all Day 75 fetuses in low concentrations. However, there was considerable variation in plasma UF concentrations among different fetuses. Mean Uf concentration in umbilical venous plasma was 79.8 ± 13.1 ng/ml and ranged from 28.8 to 452.0 ng/ml at Day 75 of pregnancy. Umbilical arterial plasma Uf concentrations were less ($P<0.07$) than those for umbilical venous plasma and averaged 43.9 ± 13.1 ng/ml with a range of 20.0 to 154.8 ng/ml. The mean venous-arterial Uf concentration difference among 17 fetuses was 36.5 ± 18.4 ng/ml with 13 positive values (range 1.5 to 297.2 ng/ml) and 4 negative values (-2.3 to -7.8 ng/ml). These data indicate that maternal Uf gains access to the fetal blood and that a portion of it is removed from the circulation during passage through fetal tissue.

Experiment 3

Earlier work (Buhi et al., 1982a) showed that ^{59}Fe from Uf is sequestered mainly in the liver. To determine whether fetal liver membranes bound Uf, an assay was conducted with the addition of increasing amounts of membrane protein in the absence of unlabeled Uf. The proportion of radiolabeled uteroferrin bound increased from 7.7% of total counts added in the presence of 75 μg of membrane protein up to 39.2% with 600 μg of membrane protein (Fig. 4A). Incubation of a constant amount of membrane protein (100 μg) and radiolabeled uteroferrin with increasing amounts of purified radioinert Uf (2.5–400 μg) substantially decreased binding compared with that in the absence of unlabeled uteroferrin (Fig. 4B). These data represent a single experimental observation. This experiment was not designed to characterize the Uf receptor but to determine whether cell membranes of liver tissue have the ability to bind Uf in a dose-dependent manner. These data, although of a preliminary nature, support that possibility.

Experiment 4

Urine samples obtained from the bladders of Day 75 fetuses contained 188.9 ± 15.9 μg

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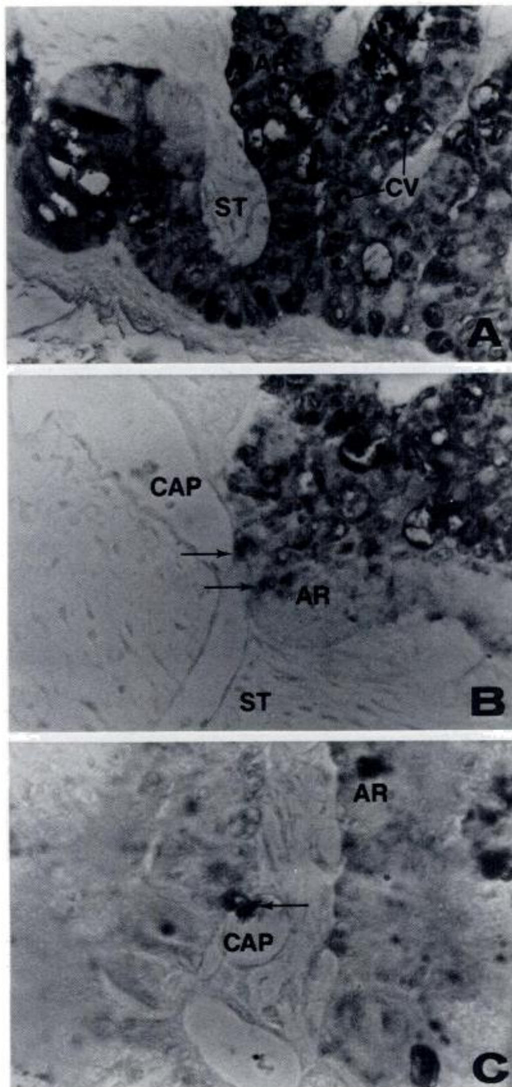

FIG. 3. Peroxidase-antiperoxidase staining for Uf in Day 75 placental tissue. *A*) Positive staining in cells of the chorioallantoic areolae (*AR*) was present within supra- and infranuclear cytoplasmic vesicles (*CV*). Note the absence of staining within the surrounding chorioallantoic stroma (*ST*). X710. *B*) Section of an areolae with Uf staining in infranuclear cytoplasmic vesicles (*arrows*) of cells adjacent to a chorioallantoic capillary (*CAP*). X800. *C*) Section of an areolae showing apparent release of Uf (*arrow*) from infranuclear cytoplasmic vesicles into a chorioallantoic capillary (*CAP*). X940.

protein/ml. Acid phosphatase activity was detected in urine of 10 of 27 fetuses and specific activity averaged 3.37 ± 1.83 μmol inorganic phosphate (P_i) released/10 min per mg protein. Values ranged from a high of 19.3 units/mg protein to a low of 0.4. Three samples of eight tested gave a discernible line of identity when tested against purified Uf using sheep antiserum against Uf by Ouchterlony double immunodiffusion analysis (Fig. 5).

Two-dimensional NEPHGE analysis of urine identified a protein with electrophoretic mobility similar to Uf in one individual and one pooled urine sample (Fig. 6), while in another individual sample Uf was not detected. Pooling was necessary to obtain enough protein for electrophoresis. Interestingly, several other polypeptides with mobilites similar to other basic proteins found in maternal uterine secretions (Basha et al., 1980) were also detectable in fetal urine (Fig. 6, *arrows*). These data

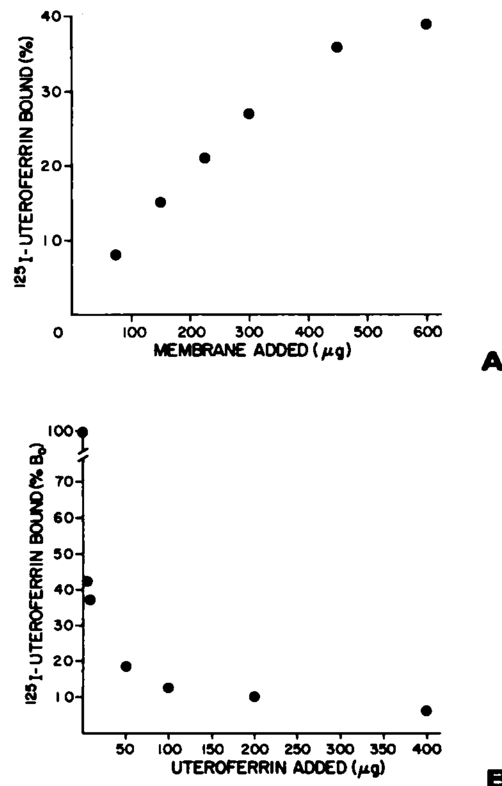

FIG. 4. *A*) Day 75 fetal liver membrane binding of ^{125}I-uteroferrin with addition of increasing quantity of membrane protein (75–600 μg). Membranes and ^{125}I-uteroferrin (20,000 cpm) were incubated at 25°C for 90 min. *B*) Day 75 fetal liver membrane binding of ^{125}I-uteroferrin with addition of increasing quantity of unlabeled Uf (2.5–400 μg). A constant quantity of membrane protein was used (150 μg). Values are expressed as percentage of the binding in control tubes which did not contain unlabeled Uf (% B_O).

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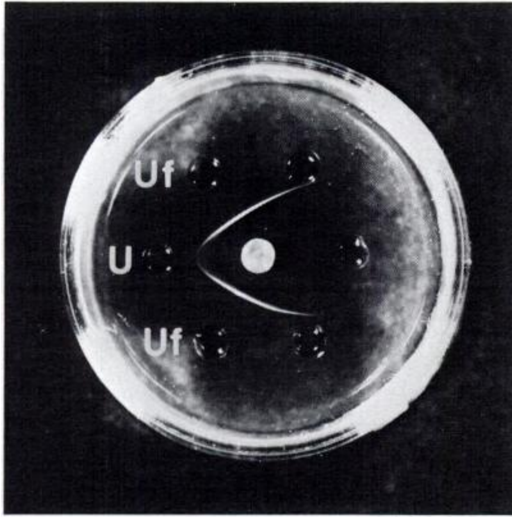

FIG. 5. Agar gel double immunodiffusion of a Day 75 fetal urine sample (*U*) and purified Uf against sheep antiserum to Uf in center well.

strongly suggest that fetal urine contains small, but highly variable amounts of Uf and possibly other proteins found in endometrial secretions.

Experiment 5

Immunocytochemical staining for Uf was observed within the epithelial cells and lumen of proximal tubules (Fig. 7A) and within the lumen of collecting ducts (Fig. 7B) of Day 75 fetal kidney tissue. Only a few cells within each proximal tubule contained positive Uf staining; however staining in these cells is indicative of Uf absorption by the proximal tubule. In collecting ducts, staining was restricted to the periphery of the lumen and this staining pattern probably resulted from concentration and adherence of protein to the luminal wall. Not all kidney tissues tested had positive staining and among tissues there appeared to be variations in relative staining intensities. Positive staining for Uf was not detected within distal tubules or within tubules of the Loop of Henle in kidney tissue. At this time there is no explanation for the absence of staining in some kidneys or the variation in staining intensity observed. Uteroferrin was not detected in fetal liver by the PAP procedure.

DISCUSSION

Immunohistochemical localization of Uf in the uterine glandular epithelium and lumen confirms that these cells are the site of synthesis and secretion of this protein as initially reported by Chen et al. (1975) who utilized an immunofluorescent antibody technique. However, these authors demonstrated a positive fluorescence for Uf in cells of the surface as well as glandular epithelium. By contrast, in the present study staining was observed only in surface epithelial cells adjacent to the chorioallantoic areolae, suggesting that the surface epithelium is not a major site of Uf synthesis and secretion. Staining of the surface epithelium adjacent to the areolae may indicate the presence of an intermediate cell type having the morphological characteristics of surface epithelium but the biosynthetic capacity to secrete Uf. In support of this, Crombie (1972) reported that uterine epithelial cells adjacent to the areolae were ultrastructurally different from interareolar uterine epithelium. In addition, Friess et al. (1981) observed large blebs budding from the apices of uterine epithelial cells bordering the areolar lumen which were not observed on uterine surface epithelium outside the areolae. Wislocki and Dempsey (1946) examined the pig placenta for Fe and acid phosphatase activity by histochemical techniques and found little reaction within the uterine surface epithelium, but abundant localization within uterine glands. Since Uf appears to be the major placental Fe transport protein in pigs (Ducsay, 1980; Roberts and Bazer, 1980; Buhi et al., 1982a) and possesses >95% of the acid phosphatase activity in uterine secretions (Bazer et al., 1975), the data of Wislocki and Dempsey (1946) in conjunction with that from the present study suggest that surface epithelium has little or no role in Uf secretion. It is possible that the fluorescence within the surface epithelium observed by Chen et al. (1975) was autofluorescence, which has been reported in this tissue by Wislocki and Dempsey (1946).

In the chorioallantois, Uf was localized in the lumen and cells of the areolae, but not other areas of the tissue. Chen et al. (1975) also demonstrated Uf in areolae and Wislocki and Dempsey (1946) observed abundant iron and acid phosphatase activity in these structures. In the present study Uf was concentrated in large and small vesicles in the supra- and infranuclear cytoplasm of cells in the areolae. In an ultrastructural study of the areolae, Friess et al. (1981) reported that cytoplasmic vesicles contained material of similar electron density

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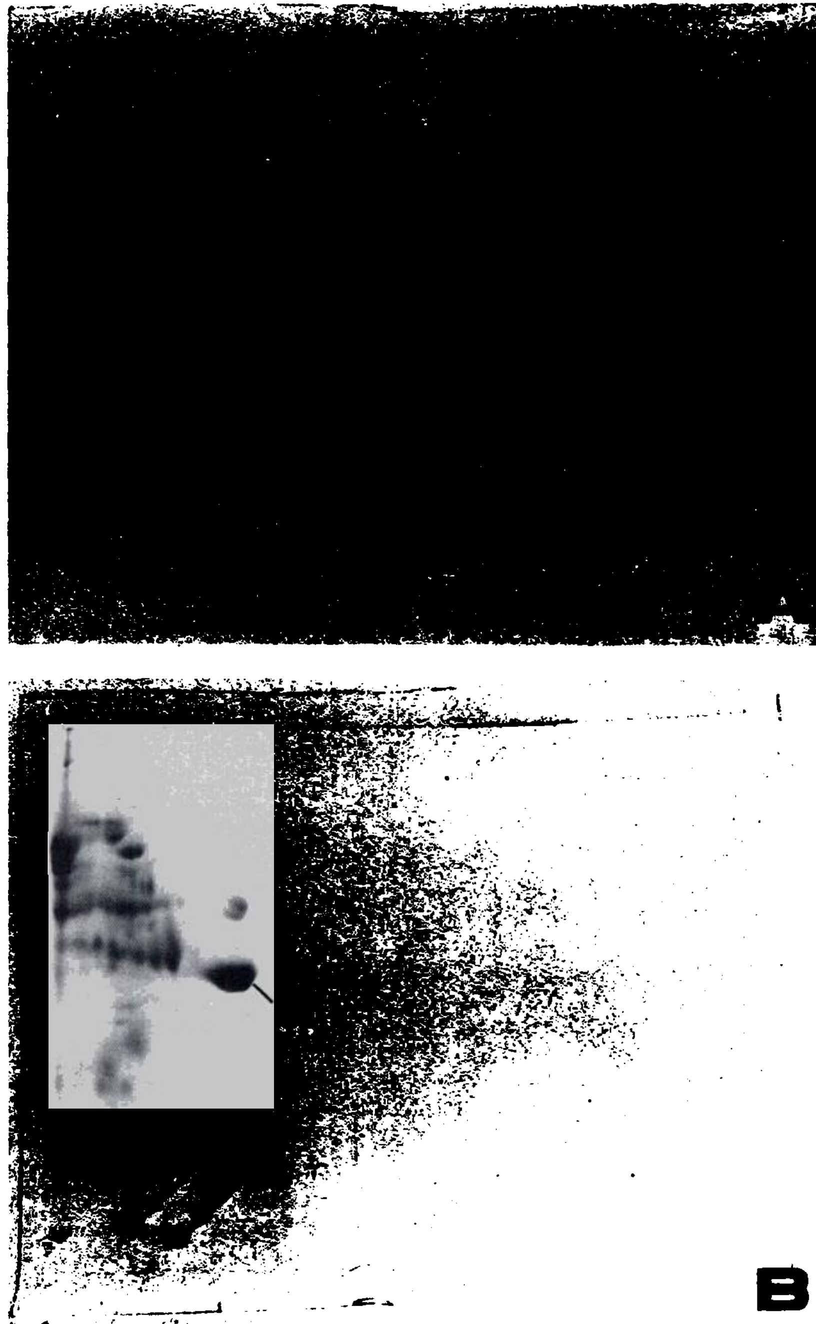

FIG. 6. *A*) Coomassie blue-stained 2D-PAGE of basic proteins (NEPHGE) in a urine sample from a Day 75 fetus. Uteroferrin (*UF*) and two other proteins (*arrows*) similar to those found in uterine secretions were present. *B*) Coomassie blue-stained NEPHGE gel of uterine secretions from Day 11 of pregnancy.

to that in the areolar lumen and suggested that these specialized chorionic epithelial cells were responsible for absorption and intracellular transport of uterine histotroph. In the present study, heavy staining for Uf was noted in vesicles within the basal cytoplasm of cells adjacent to the chorioallantoic capillaries. In some sections there appeared to be release of

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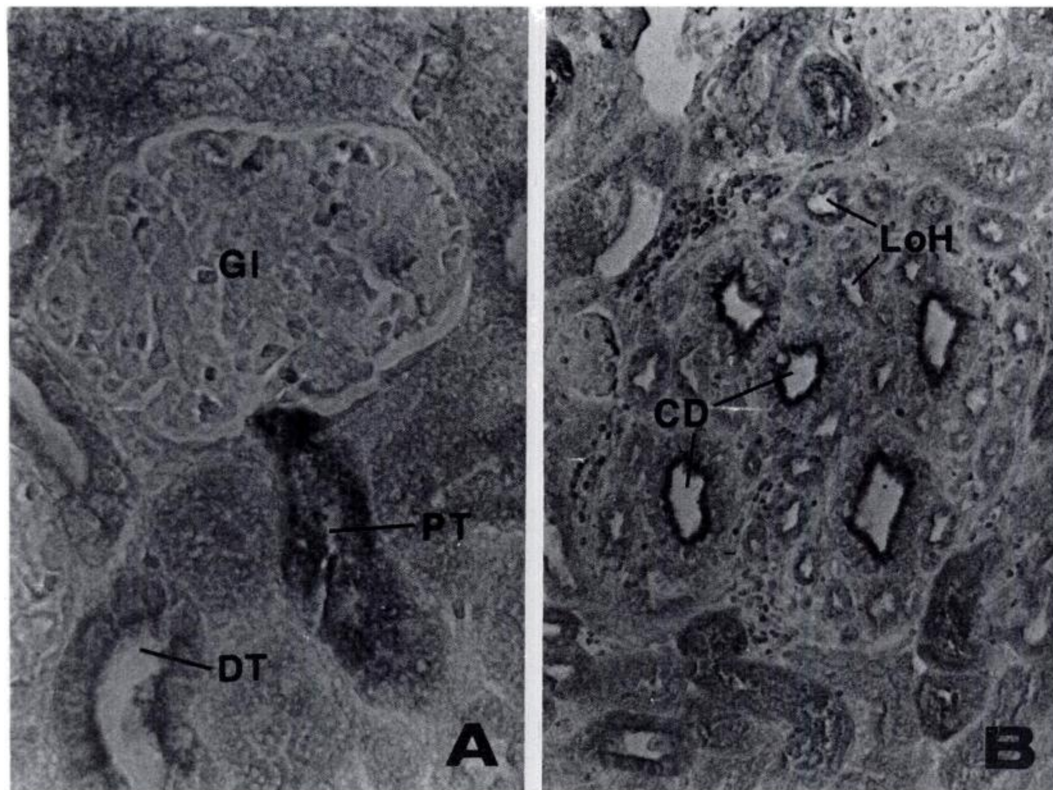

FIG. 7. Peroxidase-antiperoxidase staining for Uf in Day 75 fetal kidney tissue. *A*) Cortical area of the fetal kidney. Positive staining is present in the lumen and epithelial cells of the proximal tubules (*PT*) but not within distal tubules (*DT*) or glomerulus (*Gl*). X1600. *B*) Cross section of a medullary ray with positive staining present along the walls of the collecting ducts (*CD*). Positive staining was not observed in tubules of the Loop of Henle (*LoH*). X650.

Uf into capillaries. These observations suggest that Uf is transported by areolae from its site of secretion directly into the fetal circulation.

The chorioallantoic capillaries are especially suited for macromolecular exchange by virtue of the numerous fenestrae within the capillary endothelium (Friess et al., 1981). The mechanism of macromolecular transport across epithelial membranes of the gut (Rodewald, 1973; Smith et al., 1979) and yolk sac (Linden and Roth, 1978; Moxon and Wild, 1976) of several species has been investigated and the observations may lend understanding to the Uf uptake and transport mechanism of the areolae. Two types of transport processes were described for the tissues examined. In epithelia of rabbit and chick yolk sac and rat intestine (Linden and Roth, 1978; Moxon and Wild, 1976; Rodewald, 1973), IgG is absorbed by receptor mediated pinocytosis and transported across the cell within coated vesicles. By contrast, in the newborn pig, IgG and colostral proteins are absorbed into the intestinal epithelium by nonspecific (fluid phase) protein transport which is stimulated by high concentrations of protein in the intestinal lumen and involves uptake of substances into large cytoplasmic vacuoles (Smith et al., 1979). These vacuoles are similar to those observed within cells of the areolae in the present study, suggesting nonspecific protein transport by these structures. Chen et al. (1975) suggested that Uf transport from the areolae occurred through the chorioallantoic mesoderm and allantoic epithelium and into the allantoic sac, implying movement across several cell layers. They observed faint fluorescent staining for Uf within the cells of the chorioallantoic mesoderm; however, comparable results were not obtained in the present study. Although the reason for this discrepancy is not known, it may be related to technique differences because unlabeled antibody tech-

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niques such as the PAP method are more sensitive and specific than methods employing a detector molecule covalently bound to the antibody as in immunofluorescence (Sternberg, 1969). Thus, the chorioallantoic mesodermal fluorescence (previously reported) may have been nonspecific.

Palludan et al. (1969) administered ^{55}Fe to pregnant gilts and examined the distribution of iron within placental tissue by autoradiography. A high density of grains representing ^{55}Fe were observed in the areolae and uterine glandular epithelum and secretions. Since maternal-to-fetal iron transport is probably mediated by Uf (Ducsay et al., 1982) the data of Palludan et al. (1969) support the role of areolae in transport of Uf. In addition, Palludan et al. (1969) observed few ^{55}Fe grains in stroma surrounding the areolae which indicated that transport of iron directly across the chorioallantoic membrane into the allantoic sac was not likely.

Presence of Uf had not been examined in fetal blood prior to this study. The observed umbilical venous-arterial Uf concentration difference suggested that *1*) Uf entered the fetal circulation within the placenta, thus supporting the immunohistochemical observations of release from the areolae into chorioallantoic capillaries, and *2*) some of the Uf was removed from circulation by the fetus. It was calculated that approximately 36.0 ng of Uf was removed from each ml of blood during each pass through the fetus at Day 75 of pregnancy. Comline et al. (1979) estimated mean umbilical blood flow to be 200 ml/min per kg fetus during late pregnancy in pigs. Utilizing that umbilical blood flow value and the Day 75 fetal pig body weight of 0.322 kg reported by Ducsay (1980), Uf transfer to the fetus was calculated to be about 3.34 mg/day. Since Uf contains 0.167% Fe by weight (Buhi et al., 1982b), Fe transfer by Uf to the Day 75 fetus was calculated to be about 5.58 μg/day. Ducsay (1980) reported that total fetal-placental unit Fe increased 17.3 mg between Days 75 and 112 of gestation. This increase would require an average Fe uptake of 467 μg/day or 279 mg of Uf. Although the calculated average daily uptake of Uf from the umbilical circulation by the fetus is not adequate to account for fetal placental unit requirements it should be emphasized that this figure was a single point estimate calculated with a mean value for Uf uptake. This is especially important in light of the variation in Uf uptake among fetuses. Variation in fetal Uf uptake and variation in Uf transport to the umbilical vein among fetuses may also indicate high within fetus variability over time. In addition umbilical blood flow is highly variable (Carter, 1975). Time related, e.g. hourly, changes in these factors may allow adequate uptake of Uf from the umbilical venous blood to meet fetal Fe requirements. Chronic studies designed to investigate this concept are needed. In addition, there may be direct transfer of Fe from Uf to transferrin within the placenta itself which would increase total Fe transport by the placental circulation. An alternative mode of Fe transport during late pregnancy can not be ruled out since the rate of Uf synthesis is known to decline markedly after Day 75.

In this study, Uf was bound by fetal liver membranes, suggesting that the liver is a site of Uf uptake. In contrast, Uf was not detected in liver tissue by immunohistochemical staining. Inability to detect Uf by immunocytochemistry may be due to molecular changes in Uf resulting from receptor binding or rapid metabolism of Uf and its Fe after uptake. Hematopoesis accounts for most of the Fe requirement of the developing mammalian fetus, and the liver is the primary site of hematopoesis throughout most of fetal life (reviewed by Pearson, 1973). Previously, Bazer et al. (1975) demonstrated accumulation of Uf in the pig allantois. Loss of Fe by Uf to allantoic transferrin (Buhi et al., 1982a) and subsequent uptake of transferrin from the allantois (Ducsay et al., 1982) is followed by loss of transferrin Fe at the liver, indicating an indirect role of Uf in Fe transport. However, uptake of circulating Uf by the fetus and binding of Uf by liver membranes, as demonstrated in this study, emphasize a more direct role of Uf in Fe transport to the primary site of hematopoesis. This mechanism is supported by the work of Ducsay (1980) who administered ^{59}Fe intravenously to pregnant gilts and found high levels of radioactivity in fetal tissues (including liver) 24 h later, but little evidence for major accumulation of ^{59}Fe in the allantois. This indicated that Uf accumulation in the allantois is probably secondary to its uptake by the liver and may represent a pool of "spillover" material deposited during times of excess Uf.

Detection of activatable acid phosphatase activity and results of immunodiffusion and NEPHGE analysis demonstrated that Uf was present in fetal urine which is indicative of its

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clearance by the kidney. These results are in agreement with those of Suarez et al. (1968) who reported an electrophoretic pattern of pig urine proteins similar to that for plasma with several large molecular weight proteins being present, including albumin (65,000 daltons). The bladder of the pig is connected, by the urachus, to the allantois during most of fetal life and urine flow occurs at a high rate from about Day 45 onward (Patten, 1948; Perry and Stainier, 1962). Results of the present study indicate that Uf is cleared from fetal blood by the kidney and enters the allantois in fetal urine.

Immunohistochemical localization of Uf within collecting ducts and proximal tubules of the fetal kidney supports the described route of Uf transport to the allantois. Inability to detect Uf within the distal tubules and tubules of the Loop of Henle is probably a result of the absorptive activity of the proximal tubules in reducing the Uf concentration in the ultrafiltrate to levels below the sensitivity of the PAP procedure. This is supported by the observation that some cells of the proximal tubule epithelium contained positive staining for Uf. Proteins absorbed by the proximal tubules are degraded and breakdown products released into the peritubular space to enter surrounding capillaries (Guyton, 1976). This mechanism may act to recover Uf bound Fe for transport to transferrin within the circulation or in Fe storage since kidney tissue contains measurable ferritin (Linder et al., 1975). Detection of Uf within collecting tubules probably reflects reconcentration of the remaining Uf by removal of water from the urine.

Results from the present study support the following model for Uf transport to the developing fetus (Fig. 1). Uteroferrin within secretions of endometrial uterine glands is taken up by nonreceptor mediated (fluid phase) pinocytosis into cells of the chorionic areolae, transported to the base of the areolar cells and released into the chorioallantoic capillaries. Uteroferrin is then transported by the umbilical vein directly to the fetal liver. Much of the circulating Uf is probably bound by the liver and its Fe removed for hematopoesis and other functions. Uteroferrin not bound by the liver is partially cleared from the blood by the kidney and subsequently enters the allantoic sac along with fetal urine through the urachus. This mechanism accomplishes two purposes, i.e., direct and efficient Fe transport to the site of hematopoesis and storage of Fe in the allantoic fluid to meet subsequent requirements. It is possible that other mechanisms for iron transport from dam to conceptus exist; however, they have not yet been demonstrated.

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