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Role of Uteroferrin in Iron Transport and Macromolecular Uptake by Allantoic Epithelium of the Porcine Conceptus[1,2]

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ABSTRACT

Uteroferrin (UF) is an iron-containing, progesterone-induced glycoprotein present in allantoic fluid and uterine secretions of swine between Days 30 and 105 of gestation. The role of UF in maternal-to-fetal iron transport and uptake of macromolecules by allantoic epithelium were studied in 2 experiments.

In Experiment 1, 8 pregnant gilts were assigned to treatment on either Day 30, 60, 90 or 105 of gestation. Three additional gilts were rendered unilaterally pregnant and assigned to treatment on Day 60. All gilts received 100 μCi ^{59}Fe injected intravenously. Twenty-four h after the ^{59}Fe treatment, gilts were hysterectomized and fetal fluids and tissues collected. Uterine flushings were also collected from the nongravid uterine horn of unilaterally pregnant gilts. Fetal bone, spleen, liver, kidney and placenta accumulated ^{59}Fe, but fetal spleen was the only tissue in which total ^{59}Fe accumulation was affected ($P<0.05$) by day of gestation. Radiolabeled UF was also isolated from uterine flushings (14 cpm/mg protein) and from concentrated allantotic fluid by carboxymethyl cellulose (CMC) ion exchange chromatography.

In Experiment 2, macromolecular uptake by the allantoic epithelium, both in vitro and in vivo, was examined. Sections of allantois, chorion, amnion and fetal gut (FG) were collected from Day 60 pregnant gilts. Samples of each of the tissues were incubated in minimal essential medium (MEM) containing 1 of the following proteins labeled with fluorescein isothiocyanate (FITC): *1*) FITC-γ-globulin; *2*) FITC-uteroferrin; *3*) FITC-transferrin or *4*) FITC-γ-globulin with 10^{-4} M Na-arsenite. Uptake of these proteins by chorion and allantois, but not amnion was observed. The Na-arsenite inhibited protein uptake. When FITC-γ-globulin was introduced into the allantoic fluid on Day 60 of pregnancy, uptake by the allantois was observed.

Results of this study support the concept that uteroferrin (UF) plays a major role in iron transport to the conceptus. In addition, data indicate that the allantoic epithelium is capable of transporting proteins normally found in allantoic fluid, i.e., uteroferrin and transferrin, as well as a protein not normally found in either fetal serum or allantoic fluid, i.e., γ-globulin. Failure to detect uptake of these proteins when Na-arsenite was added to the incubation medium suggests that transport of these proteins is by an active process.

INTRODUCTION

In mammals having an invasive type of implantation, e.g., rabbit and human, the placental chorion has specific receptors for transferrin on its maternal surface (Faulk and Gailbraith, 1979; Wada et al., 1979). Transferrin has a molecular weight (MW) of about 78,000 daltons and binds a maximum of 2 Fe^{3+} atoms. The transferrin-iron complex, once bound to the chorion, is believed to be taken up by endocytosis and the iron released within the chorionic epithelium. The iron may then be bound to either placental ferritin (Wohler, 1963) or a low molecular weight iron carrier (Larkin et al., 1970). The transferrin molecule of maternal origin does not appear to enter the fetal circulation (Gitlin et al., 1964; Baker and Morgan, 1970).

In other species, for example the dog and cat, iron transport to the fetal-placental unit involves extravasation of maternal erythrocytes

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into the uterine lumen. The erythrocytes are then subjected to phagocytosis by the chorionic epithelium and/or uterine macrophages which degrade the erythrocytes. The iron released from hemoglobin is then transferred, through some mechanism, at present not understood, to the fetal circulation (Wong and Morgan, 1974). A similar process also occurs in sheep (Myagkaya and Vreeling-Sindelarova, 1976) and cattle (Bjorkman, 1969) placenta, especially within the placentomes.

Animals having diffuse, epitheliochorial placenta, e.g., the pig and horse, seem to depend upon secretion of an iron transport protein by the uterine glands. Placental iron transport in swine is believed to depend primarily on secretion of uteroferrin (UF), an iron-containing, progesterone-induced glycoprotein first described by Murray et al. (1972) and Chen et al. (1973).

Placental iron transport in swine had previously been suggested to occur via the embryotrophic route of nutrient transfer, i.e., by uterine secretions (Palludan et al., 1969). After labeling the circulating iron pool of pregnant gilts with ^{59}Fe, autoradiographic examination of the uterus revealed the highest concentration of radioactive granules associated with uterine glands, their secretions and the placental areolae (Palludan et al., 1969). These data confirmed earlier cytochemical studies which demonstrated the presence of iron in glandular epithelium and uterine lumen of pregnant swine (Wislocki and Dempsey, 1946). Uteroferrin has been detected by an immunofluorescent antibody procedure in the same regions of the placenta and pregnant porcine uterus (Chen et al., 1975). Uteroferrin is transferred across the placental areolae and can be sequestered in allantoic fluid between Days 30 and 105 of gestation, achieving maximum concentrations between Days 60 and 75 (Bazer et al., 1975). Iron and Uf accumulation in allantoic fluid follow a similar pattern (Ducsay, 1980). Available data suggest that the major role of Uf is in transplacental iron transport in swine during gestation (Schlosnagle et al., 1974; Roberts and Bazer, 1980).

The present study had 2 primary objectives. The first objective was to examine the transport of radiolabeled iron from the maternal circulation to the tissues and fluids of the fetal-placental unit and uterus. The second objective was to determine whether proteins and/or protein-iron complexes, e.g., Uf and transferrin (Tf), known to be sequestered in allantoic fluid, can be transported by the allantoic epithelium into the fetal circulation. It has been proposed that Uf in allantoic fluid represents a temporary storage form of iron (Roberts and Bazer, 1980).

MATERIALS AND METHODS

Animals

Crossbred gilts of similar age, weight and genetic background were bred at 12 and 24 h after onset of estrus using mature boars. Day of onset of estrus was designated Day 0.

Experiment 1: Placental ^{59}Fe Transport

Eight gilts were randomly assigned to treatment on either Day 30, 60, 90 or 105 of gestation. On the assigned day, 100 μCi ^{59}Fe (Amersham Corp.) in 10 ml sterile saline was injected through a polyvinyl catheter into an ear vein of each gilt. The syringe and catheter were flushed with 5 ml of sterile saline following the injection. A maternal blood sample was collected immediately from a vein in the contralateral ear at a time designated as T_0. After 24 h, gilts were hysterectomized and samples of allantoic fluid (Alf) and amniotic fluid (Amf) were collected and the volumes recorded as described by Knight et al. (1977). A maternal blood sample designated T_{24} was collected, as previously described, and fetal blood samples were obtained from the umbilical cord and fetal heart with a 22 gauge needle attached to a 1 cc syringe. Fetal spleen, liver, left kidney and a section of right tibia were dissected out, weighed and stored at −20°C until analyzed. Each placenta was treated in a similar manner. Representative samples of endometrium were also collected.

Three additional gilts were rendered unilaterally pregnant by the method of Basha et al. (1980). On Day 18 of gestation, the gilts were laparotomized and an 18 gauge needle was inserted through the uterine wall of 1 of the uterine horns near the cervix. Twenty ml of sterile saline was introduced into the uterine lumen and the bolus of fluid was massaged toward the oviduct. A small incision was made in the uterine horn near the tubouterine junction and the embryos were flushed out of the uterus. A ligation was placed around the uterine horn near the junction of the uterine horn and body and the uterine incision was closed. The uterus was then returned to the body cavity and the gilts were allowed to recover. On Day 60 of gestation, the unilaterally pregnant gilts were injected with 100 μCi ^{59}Fe and 24 h later they were hysterectomized and fetal tissues and fluids were collected as previously described. The nongravid uterine horn from each gilt was flushed with 80 ml of sterile saline and the uterine flushing recovered and volume recorded.

Radioactivity Determination

Fetal spleen, kidney and bone samples were placed in 13 × 100 mm culture tubes and counted in a Packard (Model 5130) gamma counter. Liver, placenta and endometrium were minced, and representative samples were weighed, placed in culture tubes and

counted in duplicate. One ml aliquots of Alf and Amf were pipetted into culture tubes and counted.

Pooled Alf samples from Day 60 unilaterally pregnant gilts were lyophilized and reconstituted to 0.1 the original volume with deionized water. One ml aliquots of concentrated Alf samples were counted, then mixed with an equal volume of settled carboxymethyl cellulose (CMC, Whatman), a cation exchanger, and incubated for 2 h at 4°C to absorb uteroferrin. The samples were centrifuged at 2000 X g for 10 min. The pellet was recovered, washed in 10 mM Tris, pH 8.0, resuspended and centrifuged again. The pellet was recovered and counted and then treated with a high salt buffer (10 mM Tris, 1 M NaCl, pH 8.0) to determine if counts previously associated with Uf in the CMC pellet could be removed.

Uterine flushings collected from unilaterally pregnant gilts were centrifuged at 12,000 X g for 20 min to remove cellular debris. A volume of 3 ml from each flushing was counted and loaded on a 1.5 X 90 cm Sephacryl S-200 column previously equilibrated and eluted with barbital saline (.02 M, .4 M, pH 8.0) buffer system. One ml fractions were collected, counted for radioactivity, and the protein concentration (Lowrey et al., 1951) of each fraction was determined. Acid phosphatase activity was measured as previously described (Schlosnagle et al., 1974) using *p*-nitrophenylphosphate as substrate. Coincident peaks of radioactivity and protein were examined for the presence of Uf and Tf by Ouchterlony agar gel immunodiffusion analysis with specific antiserum prepared against UF (Chen et al., 1975) and TF (Buhi, 1981). Aliquots of whole blood were collected from fetuses and gilts and radioactivity (CPM) was determined. Samples were centrifuged at 2250 X g for 10 min and plasma was recovered and counted. The remaining red blood cells were washed in cold normal saline, centrifuged and the supernatant was discarded. The process was repeated and the cells then were resuspended in 1 ml normal saline and radioactivity was determined.

Statistical Analysis

Data were analyzed by the method of least squares analysis of variance employing Statistical Analysis System (Barr et al., 1979). The pattern of ^{59}Fe accumulation in fluids and tissues was examined over days of gestation relative to variation among gilts within day of gestation.

Experiment 2: In Vitro and In Vivo Uptake of Fluorescein Isothiocyanate-Labeled Proteins by Porcine Chorioallantoic Membranes

In vitro studies. A total of 8 gilts was utilized for various aspects of this experiment. Surgery was performed on Day 60 of gestation. The uterus was exposed by midventral laparotomy and a 3–4 cm incision was made through the uterine wall, exposing the placenta. A section of chroioallantois was separated from the underlying endometrium and placed into a sterile culture dish containing minimal essential medium (MEM). The tissue was rinsed to remove excess blood and smaller sections of approximately 1 cm diameter were cut for incubations. In addition, sections of amnion and fetal small intestine were obtained and used as control tissues. After rinsing, sections of chorioallantois, amnion and fetal intestine were placed in individual 30 ml incubation flasks containing 5 ml MEM and exposed to an atmosphere of 95% O_2, 5% CO_2 in a Dubnoff Metabolic Shaker at 37°C for 30 min for tissue equilibration.

At the end of the equilibration period, chorioallantois and amnion tissues were transferred to fresh MEM containing either: *1)* MEM alone as a control; *2)* fluorescein isothiocyanate (FITC)-labeled γ-globulin; *3)* FITC-Uf or *4)* FITC-Tf. Fetal small intestine was incubated either in MEM alone or with FITC-γ globulin.

The FITC-γ globulin was prepared as described by Lecce (1966). Porcine γ-globulin (United States Biochemical) was added to 0.5 M carbonate-bicarbonate buffer, pH 9.0, to give a concentration 40 mg/ml. The γ globulin solution was then mixed with 1 g FITC (10% on celite, United States Biochemical) and stirred for 16 h at 4°C. The solution was stirred, centrifuged at 2,000 X g for 15 min and the supernatant removed and dialyzed against 3.5 l of 10 mM Tris-HCL, pH 8.0, at 4°C for 7 days with daily changes of buffer. Uteroferrin, purified by the procedure of Chen et al. (1973), and Tf, purified as described by Buhi (1981) were labeled with FITC using the same procedure.

Upon completion of the incubation with FITC-labeled proteins for 30 min, the tissue sections were washed in fresh MEM, placed in 10% buffered formalin for 16 h and then processed through increasing concentrations of alcohol and xylene. After fixation, the tissues were blocked in paraffin and 5 μ sections were cut, placed on slides and dried. The paraffin was subsequently removed and the sections were mounted in a nonfluorescent mountant (Permount).

Sections of chorion and amnion were also incubated with FITC-γ-globulin in an anaerobic atmosphere (N_2) or in MEM with FITC-γ-globulin and 10^{-4} M Na-arsenite. Previously described incubation conditions and tissue preparation methods were utilized. Results of these incubations would indicate whether or not protein uptake was an active process.

A modification of the in vitro method outlined above was used with a portion of the chorioallantois. Instead of being placed directly into an incubation flask, a section of chorioallantois was placed on a modified Ussing chamber (Ussing and Zerahn, 1951). This consisted of a lucite chamber filled with MEM, but divided by the section of chorioallantois. A mixture of 95% O_2, 5% CO_2 was bubbled through MEM on both sides of the chamber for a 30 min equilibration period at 37°C. Then, FITC-γ-globulin was added to either the chorionic or allantoic side of membrane in a concentration of 5 mg/ml. The incubation was terminated after 30 min and tissues were washed, fixed and sectioned as described earlier, to determine if uptake of γ-globulin was preferential by either the chorion or allantois.

In vivo studies. Porcine uterine secretions, fetal serum and allantoic fluid do not contain γ-globulins (Lecce, 1966; Buhi, 1981). Therefore, this part of the study was to determine whether or not the allantoic epithelium is capable of transporting porcine γ-globulin into the fetal circulation. Porcine γ-globulin was chosen since it is not normally present in fetal serum and because it is normally absorbed by gut epithelium of the neonate; allantoic epithelium is derived from an evagination of the fetal hindgut.

On Day 60 of gestation, the gilt was laparotomized to expose the uterus and individual allantoic fluid sacs

were isolated. A 20 gauge needle was inserted through the uterine wall into an individual allantoic sac. Correct placement was assured by insertion near the end of each allantoic sac and away from the amniotic sac and withdrawal of a small amount of allantoic fluid into a 20 ml syringe. Once needle placement was confirmed, either 400 mg FITC-γ-globulin in 20 ml or 100 mg unlabeled γ-globulin in 10 ml sterile saline was injected into the allantoic sac. The position of each injected conceptus was noted, the uterus returned to the body cavity and the incision closed. The gilts were hysterectomized 24 h later and sections of chorioallantois from conceptuses which had FITC-γ-globulin injected into the allantoic sac were fixed and sectioned. Blood samples were collected from fetuses which had unlabeled γ-globulin injected into the allantoic fluid compartment and from uninjected control fetuses. In addition, allantoic fluid volumes were measured. Maternal blood was also collected. Blood samples were refrigerated overnight, centrifuged at 2000 × g for 10 min and serum was collected and frozen at −20°C until analyzed. Serum samples were tested for the presence of γ-globulin by Ouchterlony agar gel immunodiffusion analysis against specific antibody prepared against porcine γ-globulin.

Iodination of Transferrin

Iodination of Tf was by the method of Markwell (1978) using Iodo-Gen (Pierce Chemicals). Porcine Tf (0.4 to 1.1 mg) in 1 ml 0.02 M sodium barbital buffer, pH 7.5, containing 0.4 M NaCl, was added to an Iodo-Gen coated tube (100 μg) previously rinsed with labeling buffer. Carrier-free $Na^{125}I$ (Amersham Corp.) (0.5 to 1.5 mCi) was added and the tube shaken gently for a few seconds every minute for 10 to 15 min. The ^{125}I-Tf was separated from unreacted ^{125}I by gel filtration on Sephadex G-50 or G-100. To determine if Tf could cross the placental barrier, ^{125}I-Tf was introduced into the maternal circulation of a gilt on Day 58 of pregnancy. Twenty-four h later fetal blood, allantoic fluid and amniotic fluid from 6 conceptuses were examined for the presence of ^{125}I-Tf by trichloroacetic acid precipitation and immunoprecipitation with rabbit anti-porcine Tf antiserum. Radioactivity was determined in the precipitate and supernatant after treatment of each sample.

Photomicroscopy

Tissue sections were observed and photographed using a Zeiss fluorescence microscope. Photographs were made on Kodak Plus X film with bracketed exposure times of 1, 2 and 3 min through a 10 × occular and either a 27 × or 54 × oil objective.

RESULTS

Experiment 1: Placental ^{59}Fe Transport

Total ^{59}Fe accumulation in fetal tissues 24 h after introduction of 100 μCi ^{59}Fe into the maternal circulation is summarized along with tissue weights in Table 1. Total ^{59}Fe ($\bar{X} \pm$ SEM) in fetal liver increased substantially from Day 30 (1809 ± 267 CPM) to Day 60 (56,551 ± 8083 CPM), but changed very little thereafter. The overall day trend was significant ($P<0.01$). The increase in total ^{59}Fe in fetal liver was closely associated with the increase in weight ($P<0.01$) of the fetal liver and not with major changes in CPM of ^{59}Fe per g of liver. The same trend was also observed for total ^{59}Fe in the placentae.

Fetal spleen and kidney tissues could not be obtained on Day 30 of gestation. However, total ^{59}Fe in kidney increased, but not significantly, between Days 60 and 105 of gestation. Changes in total ^{59}Fe in the spleen were significant ($P<0.05$) over the days studied. The increase in total ^{59}Fe in fetal spleen and kidney was associated with increased weights of those tissues and not with increased concentrations of ^{59}Fe/g in the respective tissues.

Data on ^{59}Fe concentration and total ^{59}Fe in bone and endometrium are summarized in Table 1. The ^{59}Fe concentration changes during gestation were significant ($P<0.05$) for bone and endometrium. For bone, only ^{59}Fe/g was determined for femur; however, increased values after Day 60 were consistent with increased hematopoeisis in bone marrow. For endometrium, ^{59}Fe/g was higher on Days 30 and 60 of gestation than Days 90 and 105. It has been demonstrated previously that Uf secretion by endometrium decreases after Day 60 (Basha et al., 1979); therefore, the decrease in ^{59}Fe/g of tissue was consistent with previous data.

The major portion of radioactivity in maternal blood at T_0 was associated with the plasma fraction while the erythrocyte fraction contained the majority of counts at T_{24} (Table 2). This difference was attributed to ^{59}Fe incorporation into maternal red blood cells and uptake by maternal tissues. The amount of radiolabeled iron in whole fetal blood, plasma and erythrocytes was maximal on Day 60 and declined to Days 90 and 105 (Table 3). The major proportion of counts was associated with the erythrocyte fraction indicative of incorporation of transferred ^{59}Fe into erythrocytes and, presumably, fetal hemoglobin.

Radioactivity in allantoic fluid was very low; however, when samples of Day 60 allantoic fluid were concentrated, significant amounts of ^{59}Fe were detectable (Table 4). Ion exchange chromatography on carboxymethyl cellulose (CMC) demonstrated that essentially all of this radioactivity was retained by the CMC, but could be eluted with 1 M NaCl (Table 4). Since Uf is the only known basic iron-containing

TABLE 1. Changes in ^{59}Fe concentrations (CPM/g) and total ^{59}Fe of fetal liver, spleen, placenta, kidney, bone and endometrium at selected states of gestation ($\bar{X}$ ± SEM).

Item	30	60	90	105
Liver (N)				
N[a]	23	16	22	15
Weight, g†	0.3 ± 0.01	6.0 ± 0.5	17.1 ± 0.8	19.5 ± 0.9
CPM/g	5498 ± 697	8838 ± 614	4304 ± 127	3115 ± 134
Total CPM*	1809 ± 268	56551 ± 8083	72972 ± 3440	61637 ± 3815
Total CPM, (%)[b]	0.0008 ± 0.001	0.026 ± 0.004	0.033 ± 0.002	0.028 ± 0.002
Spleen (N)				
Weight , g†	...	0.07 ± 0.01	0.8 ± 0.04	0.9 ± 0.05
CPM/g	...	7045 ± 863	6977 ± 260	6073 ± 384
Total CPM**		456 ± 57	5388 ± 269	5240 ± 529
Total CPM, (%)		0.0002 ± 0.00002	0.002 ± 0.0001	0.003 ± 0.0002
Kidney (N)				
Weight, g**	...	0.8 ± 0.1	3.1 ± 0.2	2.7 ± 0.2
CPM, g**	...	353 ± 20	180 ± 15	155 ± 7
Total CMP	...	283 ± 35	458 ± 20	562 ± 36
Total CPM, (%)		0.00013 ± 0.00002	0.0002 ± 0.00002	0.0003 ± 0.00002
Placenta (N)				
Weight, g**	29.0 ± 2.1	162.8 ± 0.813	187.4 ± 16.7	172.7 ± 14
CPM/g	359 ± 40	210 ± 12	190 ± 15	173 ± 19
Total CPM	11591 ± 7915	35151 ± 5540	33536 ± 3973	27004 ± 1617
Total CPM, (%)	0.005 ± 0.0007	0.016 ± 0.0002	0.015 ± 0.002	0.012 ± 0.001
Bone (N)				
CPM/g†	...	467 ± 53	1146 ± 67	1067 ± 67
Endometrium (N)				
CPM/g**	1058 ± 328	1658 ± 368	512 ± 34	888 ± 394

[a]N—number of observations per day, but for endometrium there were 3 per day.

[b]Total CPM per organ divided by total CPM introduced into maternal circulation, i.e., approximately 222,000 CPM multiplied by 100 and expressed as percent.

*Day trend significant; ($P<0.10$).

**Day trend significant; ($P<0.05$).

†Day trend significant; ($P<0.01$).

TABLE 2. Levels of ^{59}Fe in maternal whole blood, plasma and erythrocytes at T_0 and T_{24} gestation.[a]

Day of gestation	Time	Whole blood (cpm/ml)	Plasma (cpm/ml)	Cells (cpm/ml resuspended cells)
30	T_0	4113 ± 411	6635 ± 1243	133 ± 130
	T_{24}	1453 ± 444	740 ± 231	2241 ± 1893
60	T_0	6096 ± 1800	10144 ± 4994	164 ± 103
	T_{24}	1235 ± 795	560 ± 519	1346 ± 594
90	T_0	3881 ± 1539	7929 ± 1240	167 ± 24
	T_{24}	1468 ± 546	281 ± 80	982 ± 81
105[b]	T_0	...	...	...
	T_{24}	...	...	...

[a]Mean ± SEM.

[b]Samples hemolyzed.

TABLE 3. Accumulation of ^{59}Fe in fetal whole blood, plasma and erythrocytes during gestation.[a]

Day of gestation	N[b]	Whole blood (cpm/ml)	Plasma (cpm/ml)	Cells (cpm/ml resuspended cells)
30		...	...	...
60	16	3349 ± 436	275 ± 52	964 ± 171
90	22	1479 ± 52	135 ± 15	588 ± 48
105	16	1123 ± 97	106 ± 39	591 ± 37

[a]Mean ± SEM.

[b]N—number of observations.

TABLE 4. Levels of ^{59}Fe in concentrated allantoic fluid from Day 60 unilaterally pregnant gilts.[a]

No. of samples	3
cpm/ml	29 ± 11
CMC + fraction (cpm)	20 ± 10
Counts remaining after high salt wash (cpm)	2 ± 2

[a]Mean ± SEM.

protein in allantoic fluid, the majority of ^{59}Fe in allantoic fluid seemed to be associated with uteroferrin.

In an attempt to determine whether any ^{59}Fe-protein complexes other than uteroferrin were present in uterine secretions of unilaterally pregnant pigs, uterine flushings were obtained from the nongravid uterine horn on Day 60 of gestation from gilts that had received 100 μCi ^{59}Fe 24 h prior to collection of uterine secretions. The uterine flushings were analyzed for acid phosphatase activity, protein and ^{59}Fe content. The results are summarized in Table 5. The uterine flushings contained about 14 CPM of ^{59}Fe/mg protein and a total ($\bar{X}$ ± SEM) of 11,557 ± 2218 CPM. To determine if ^{59}Fe was associated with Uf, total ^{59}Fe-labeled uterine flushings were subjected to Sephacryl S-200 column chromatography. A representative elution profile is shown in Fig. 1. Two major protein peaks were observed which contained significant amounts of radioactivity. The first peak centered around Fraction 81 had an elution position similar to that of Tf, i.e., an apparent molecular weight of 70,000 to 80,000 daltons, whereas the second peak (Fraction 95)

TABLE 5. Acid phosphatase activity, protein content and ^{59}Fe content per horn in uterine flushings from the nongravid horn of unilaterally pregnant gilts.[a]

No. of flushings	3
Acid phosphatase concentrations (μmol Pi/min/ml)	100.8 ± 29.3
Total acid phosphatase (μmol Pi/min/flush)	5,442 ± 2,380
Protein concentration (mg/ml)	17.0 ± 1.0
Total protein (mg)	855 ± 170
^{59}Fe concentration (cpm/ml)	232 ± 28
Total ^{59}Fe (cpm)	11,557 ± 2,218

[a]Mean ± SEM.

eluted at a volume coincidental with that for Uf, i.e., a MW of about 35,000 daltons. The peak elution volumes of Tf and Uf had previously been determined by column calibration with purified iodinated Tf and Uf as noted by the *arrows* (Fig. 1). Ouchterlony agar gel immunodiffusion revealed that proteins in each peak cross-reacted with rabbit anti-Uf (Fig. 2A), but not antiserum to Tf (Fig. 2B). Furthermore, acid phosphatase activity was detected in fractions in each of the peaks. It has been reported previously that uteroferrin exists as a monomer and as a dimer in low ionic strength buffers (Chen et al., 1973). These data support the presence of Uf, but not Tf in uterine secretions from the nongravid uterine horn of unilaterally pregnant gilts. Based on data presented by Basha et al. (1980), proteins secreted by endometrium from the gravid and nongravid uterine horns of unilaterally pregnant pigs are qualitatively identical.

In a related experiment, ^{125}I-Tf was introduced into the maternal circulation to determine if it could cross the placental barrier and act to transport iron to the pig conceptus. Twenty-four h after ^{125}I-Tf injection into the maternal blood, radioactivity could be detected in allantoic fluid, amniotic fluid and fetal umbilical

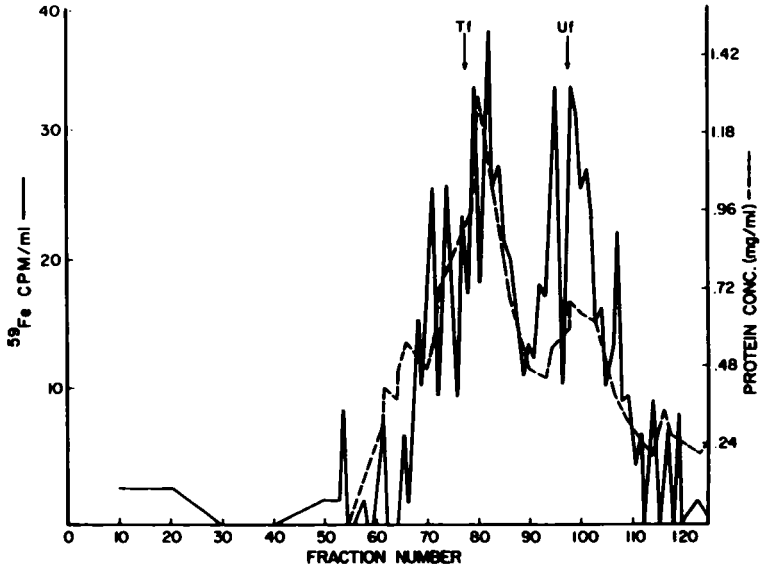


FIG. 1. Sephacryl S-200 chromatography of a uterine flushing collected from a Day 60 unilaterally pregnant gilt injected with ^{59}Fe. Protein concentration (---) and ^{59}Fe content (——) are plotted against elution volume (1 ml fractions). The column (1.5 X 90 cm) was previously calibrated with purified iodinated porcine transferrin (*Tf*) and uteroferrin (*Uf*). Expected elution volumes of these proteins are indicated by *arrows*.

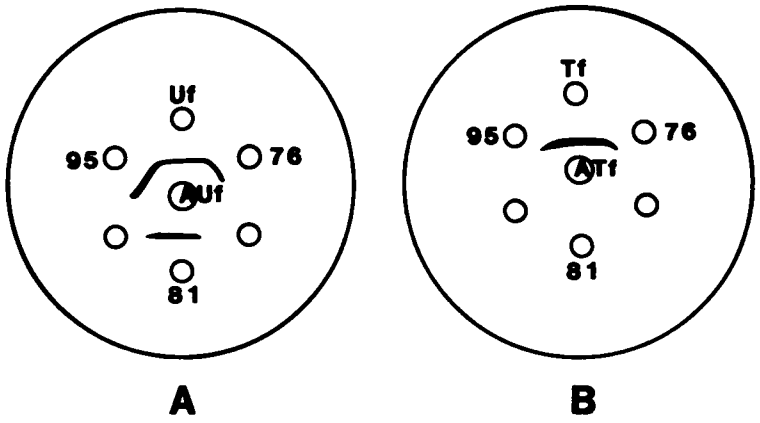


FIG. 2. *A*) Ouchterlony immunodiffusion analysis of protein fractions (see Fig. 1) eluted from a Sephacryl S-200 column loaded with uterine flushing from a Day 60, unilaterally pregnant gilt. Uteroferrin antiserum (*AUF*) was placed in the center well. Wells labeled *76*, *81* and *95* represent those respective protein fractions and the well labeled *Uf* represents uteroferrin standard. Single precipitin lines formed against protein in fractions 76, 81 and 95 indicated the presence of uteroferrin in each of the fractions. *B*) Antiserum to transferrin (*ATF*) was placed in the center well. Wells labeled *76*, *81* and *95* represent those respective protein fractions and the well labeled *Tf* represents porcine transferrin standard. No precipitin lines were formed against protein in any of the fractions which is indicative of the absence of transferrin.

cord blood. These samples were subjected to both 50% (w/v) trichloroacetic acid and rabbit anti-transferrin antibody to achieve precipitation of ^{125}I-Tf. No precipitation of radioactivity was achieved by either method. These results suggest that the ^{125}I in the fetal fluids and cord blood was no longer associated with Tf or peptides of significant (5,000 to 10,000 M_r) size which would be precipitated by trichloroacetic acid. These results further indicate that maternal Tf does not cross the placental barrier.

Experiment 2: In Vitro and In Vivo Uptake of Proteins by Porcine Chorioallantois

In vitro studies. The gut of neonatal pigs is known to take up γ-globulins by an energy dependent form of pinocytosis (Lecce, 1966; Clarke and Hardy, 1971). Since Uf (Bazer et al., 1975) and Tf (Buhi, 1981) are known to be present in allantoic fluid and since the allantois is derived embryologically from gut epithelium, this study was designed to determine whether or not the allantois is capable of uptake of macromolecules. Uptake of FITC-γ-globulin and its concentration in some, but not all epithelial cells, of Day 60 fetal gut is indicated in Fig. 3A. These areas of specific uptake are in contrast to nonspecific fluorescence observed in sections of amnion incubated with FITC-γ-globulin (Fig. 3B). No fluorescent droplets were observed in any cells within the amniotic epithelium.

Uptake of FITC-labeled proteins was difficult to recognize in chorionic epithelium because of heavy nonspecific fluorescence seen in control sections. However, when chorionic areolae were examined, bright regions of fluorescence, not seen in control sections, were observed for FITC labeled γ-globulin, Uf and Tf. These observations suggest that uptake of all of these proteins occurs by a similar mechanism. The presence of FITC-labeled γ-globulin (Fig. 4A), Uf (Fig. 4B) and Tf (Fig. 4C) in epithelial cells of the allantois was also demonstrated.

Sections of chorioallantois placed in a modified Ussing chamber permitted incubation with only the allantoic side of the membrane in contact with FITC-γ-globulin. Uptake of the FITC-γ-globulin was observed (Fig. 5C). However, when sections of chorioallantois were incubated under anaerobic conditions or in the presence of 10^{-4} M Na-arsenite, uptake of FITC-γ-globulin was not observed (Fig. 5B). Incubation of intestine from neonatal pigs in the presence of 10^{-4} M Na-arsenite or under anaerobic conditions also prevented FITC-γ-globulin uptake (Lecce, 1966).

In vivo studies. The first part of this study involved introduction of FITC-γ-globulin into

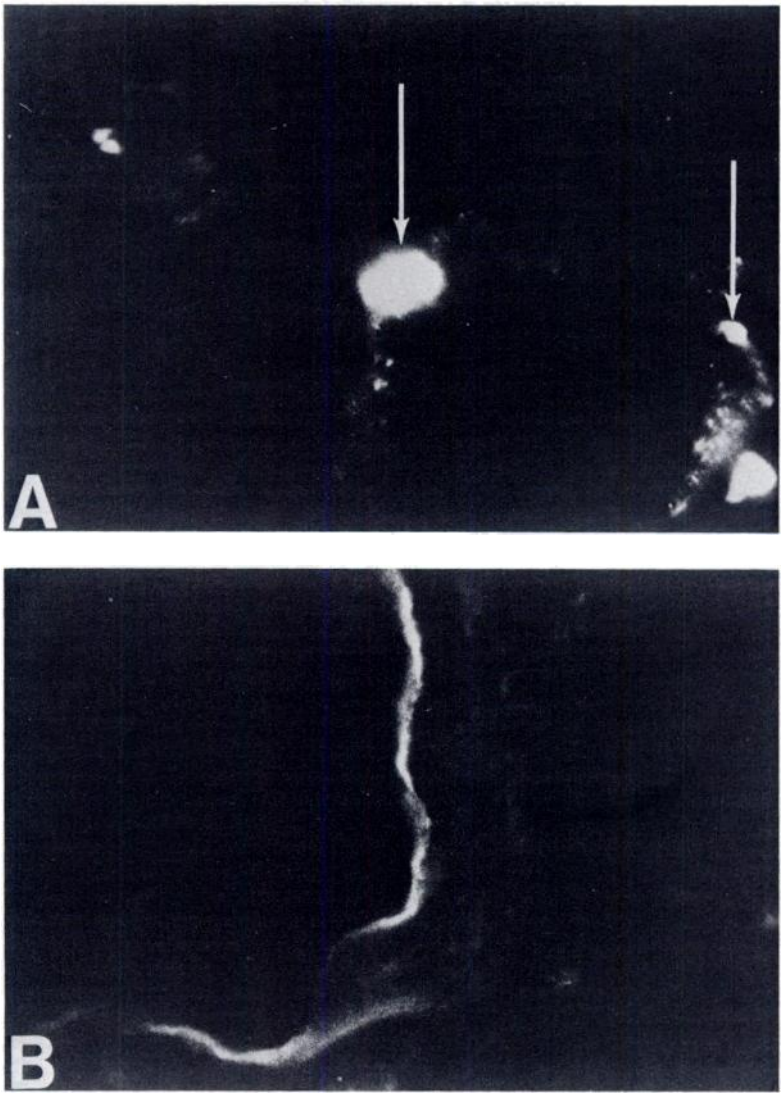
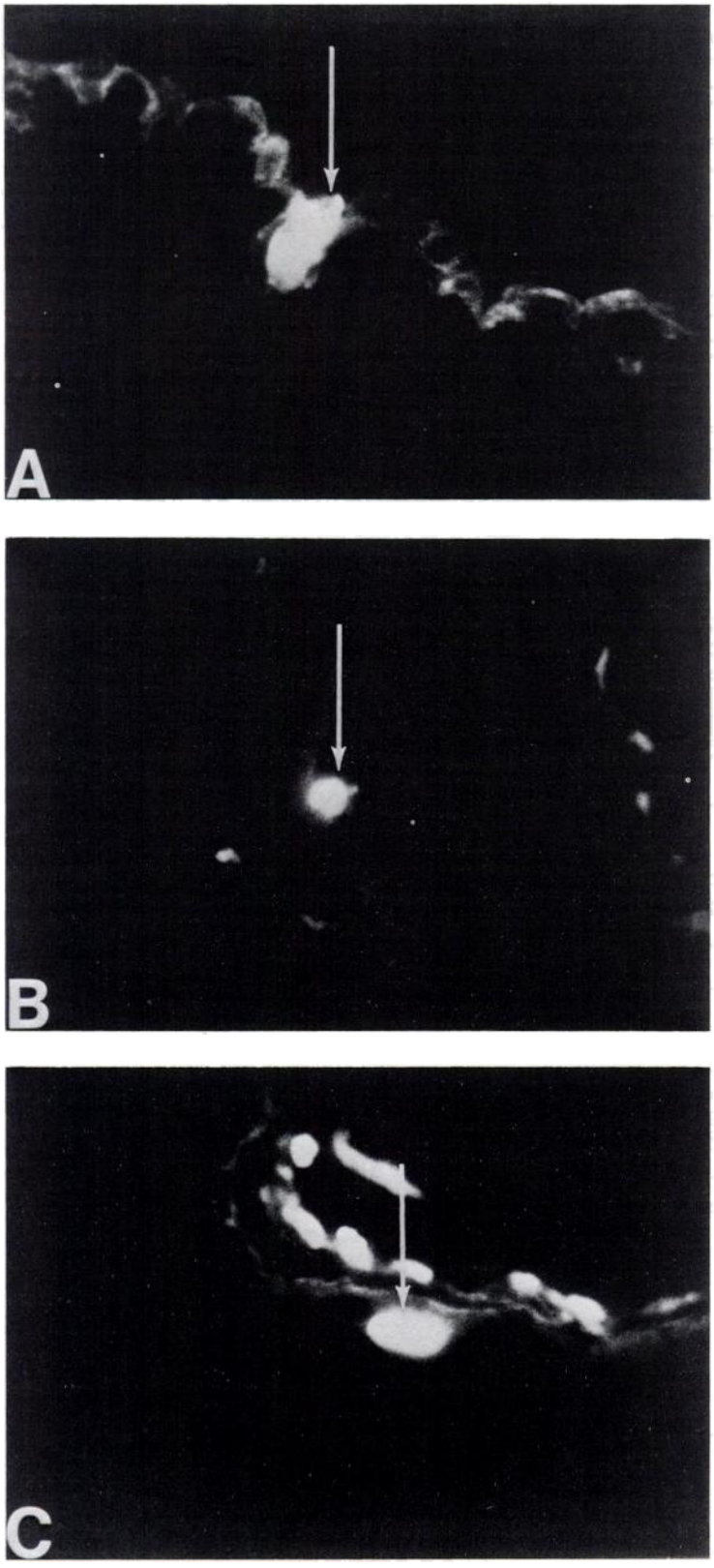


FIG. 3. *A*) Section of Day 60 fetal gut incubated in vitro with fluorescein-labeled γ-globulin (FγG) at 37°C for 30 min in MEM. *Arrows* point to droplets of FγG within epithelium. X270. *B*) Section of Day 60 amnion incubated in vitro with fluorescein-labeled γ-globulin (FγG) at 37°C for 30 min in MEM. No adsorption of FγG was observed. X270.

the allantoic fluid compartment of Day 60 pregnant gilts to determine its uptake by allantoic epithelium in vivo 24 h later. The FITC-γ-globulin is not a normal component of allantoic fluid, but served as a means of assessing protein uptake. Data from this study confirmed the in vitro results since uptake of the FITC-γ-globulin by the allantois was observed (Fig. 5A).

In the second part of this study, unlabeled γ-globulin (100 mg/ allantoic sac) was introduced into the allantoic fluid compartment of 2 Day 60 pregnant gilts to determine whether the γ-globulin reached the fetal circulation. Since fetal piglet serum is normally devoid of γ-globulin (Lecce, 1966), this protein served as a useful probe to study macromolecular uptake by the allantoic epithelium and transport into the fetal circulation.

Two of 6 treated fetuses had detectable



γ-globulin in serum samples based upon Ouchterlony immunodiffusion analysis, while γ-globulin was not detected in serum collected from control fetuses. A representative Ouchterlony plate with fetal serum samples from 1 of the gilts is shown in Fig. 6. The combined results of Experiment 2 are summarized in Table 6.

DISCUSSION

It has been demonstrated that Uf crosses the placenta (Chen et al., 1975) and is sequestered temporarily in allantoic fluid, but not in amniotic fluid (Bazer et al., 1975). The Uf-mediated iron transport from allantoic fluid into the fetal circulation was hypothesized to occur by uptake of iron or protein-iron complexes by allantoic epithelium. Based upon this concept, it was postulated that administration of ^{59}Fe to the dam would result in accumulation of radiolabeled iron in allantoic fluid and fetal and placental tissues.

Tissue and blood ^{59}Fe concentrations observed in fetuses in Experiment I are comparable to those obtained in earlier studies (Hoskins and Hansard, 1964; Palludan et al., 1969). That is, they found ^{59}Fe accumulation in placenta and liver to be much greater than that in spleen, kidney, bone and plasma; however, they did not evaluate allantoic fluid for radiolabeled iron. In this study, ^{59}Fe in allantoic fluid was present in low concentrations, but that which was recovered appeared almost exclusively associated with uteroferrin. There are several possible explanations for the low amount of ^{59}Fe-Uf in allantoic fluid. First, since only 100 μCi of ^{59}Fe was injected into the maternal circulation, the amount of ^{59}Fe-Uf produced within 24 h may be small. If so, it would become diluted in the large volume of allantoic fluid found in pregnant pigs at Day 60 of gestation (Goldstein et al., 1980). Second, the ^{59}Fe-Uf produced by the uterine endometrium may initially enter the fetal circulation via the placental areolae and give up a majority of the iron to liver and spleen. The ^{59}Fe-Uf not degraded by fetal tissues might then be cleared through the kidney and enter the allantoic sac via the urachus. The ^{59}Fe-Uf "spillover" into the allantoic sac might be rather small considering the limited mass of ^{59}Fe introduced into the maternal circulation.

Unilaterally pregnant pigs were utilized to determine the form of ^{59}Fe in uterine secretion of pregnant pigs. It had been established previously (Basha et al., 1980) that endometrial secretions from the gravid and nongravid uterine horns of unilaterally pregnant pigs are qualitatively indistinguishable. Acid phosphatase activity and protein content in uterine flushings from unilaterally pregnant gilts in Experiment I (Table 5) are comparable to those reported by Basha et al. (1980). However, a total of only 11,500 CPM ^{59}Fe was obtained. This represents a small fraction of that found even in a single fetus at Day 60, i.e., at least 100,000 CPM, after the gilt received 100 μCi intravenously. Radiolabeled iron was not found to be present as free ^{59}Fe nor bound to any macromolecule other than Uf in uterine secretions. An explanation for the relatively low ^{59}Fe-Uf in the uterine flushings may be due to either a rapid turnover of Uf in uterine secretions or the fact that most of the ^{59}Fe-Uf remained trapped within the uterine glands by 24 h after ^{59}Fe administration. It is also possible that ^{59}Fe-Uf synthesis and/or secretion is limited if the product is not taken up by the placental areolae.

It has been established that the areolae of the pig chorioallantois are the primary sites of absorption of endometrial secretory products (Brambell, 1933; Palludan et al., 1969; Chen et al., 1975). Although the mechanism of transport is not known, Uf produced by the uterine glands of pigs accumulates in the allantoic sac. The Uf may be: *1*) transported across the chorionallantoic membranes, as well as the mesenchymal tissue separating those 2 membranes, and into the allantoic fluid or *2*) transported from the areolae into the fetal circulation, cleared through the kidney and moved into the allantoic sac via the urachus. Once in the allantoic sac, results from Experiment 2

FIG. 4. *A*) Section on Day 50 areolae incubated in vitro with fluorescein-labeled γ-globulin (FγG) at 37°C for 30 min in MEM. *Arrow* indicates droplet of FγG within epithelium. X270. *B*) Section of Day 60 allantois incubated in vitro with fluorescein-labeled uteroferrin (Uf) at 37°C for 30 min in MEM. Absorption of Uf indicated by *arrow*. X270. *C*) Section of Day 60 allantois incubated in vitro with fluorescein-labeled transferrin (Tf) at 37°C for 30 min in MEM. Absorption of Tf indicated by *arrow*. X270.

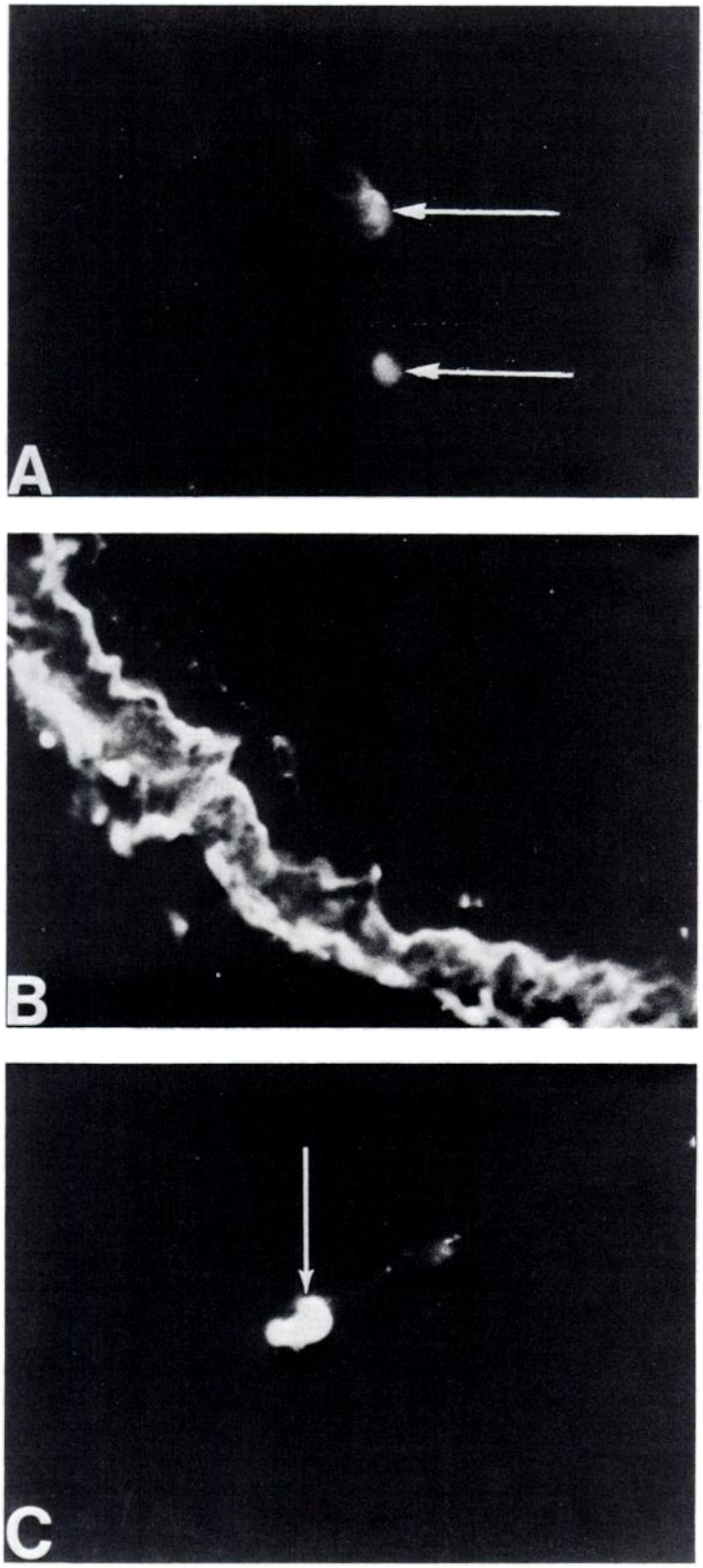


FIG. 5. *A*) In vivo absorptive capacity of Day 60 allantois. Section of allantois from a Day 60 pregnant gilt which had fluorescein-labeled γ-globulin (FγG) injected into the allantois was obtained at hysterectomy. *Arrows* point to droplets of FγG within allantoic epithelium. X 270. *B*) Effect of 10^{-4} M sodium arsenite on absorption of fluorescein-labeled γ-globulin (FγG) by Day 60 allantois. This section of allantois was preincubated in vitro at 37°C with 10^{-4} M sodium arsenite in MEM for 30 min. Following preincubation, allantoic tissue was transferred to fresh MEM containing FγG for 30 min. No droplets of FγG were observed within epithelium. X 540. *C*) Section of Day 60 allantois incubated in vitro in an Ussing chamber with fluorescein-labeled γ-globulin on the epithelial side of the membrane. After incubation at 37°C in MEM, FγG was present within the epithelium (*arrow*). X 270.

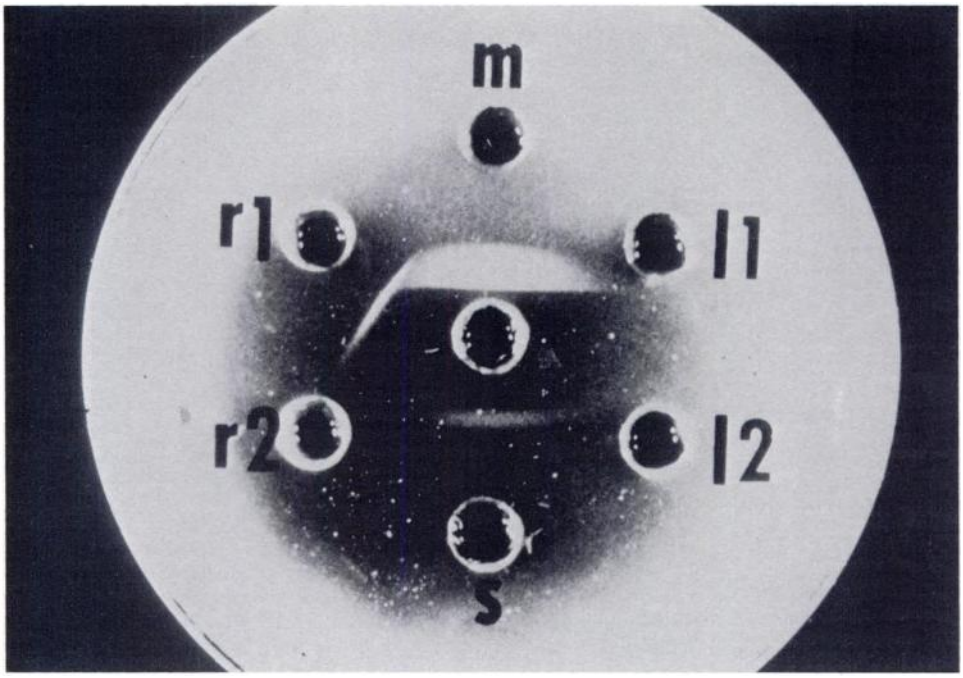


FIG. 6. Ouchterlony immunodiffusion analysis of serum from fetuses (r_1 and r_2) which had porcine γ-globulin introduced into allantoic fluid in vivo and uninjected control fetuses (l_1 and l_2). The center well contains antiserum to porcine γ-globulin. Maternal serum and γ-globulin standard (*S*) were also tested. A single precipitin line between R_1 and *M* indicated the presence of γ-globulin in serum from fetus R_1 and maternal serum.

TABLE 6. Absorption of fluorescein-tagged proteins by porcine chorion (C), allantois (AL), amnion (AM) and fetal intestine (FI) in vitro and in vivo.

	In vitro						In vivo
Tissue	FγG	FUF	FTF	FγG Ussing chamber	FγG 10^{-4} M Na-arsenite	FγG N_2 atmosphere	FγG
C	+	+	+	+	–	–	0
AL	+	+	+	+	–	–	+
AM	–	–	–	–	–	–	0
FI	+	0	0	0	0	0	0

+ – uptake.

– – lack of observable uptake.

0 – not tested.

indicate that the allantoic epithelium can transport macromolecules by an active cellular process that may be analagous to that of γ-globulin uptake by the gut of neonatal piglets (Lecce, 1966; Clark and Hardy, 1971). Recent results (Buhi, 1981) suggest that Uf may give up its iron to fetal Tf in the allantoic sac. The Tf in allantoic fluid can then be transported across the allantoic epithelium and into the fetal circulation, while apo-Uf is rapidly degraded in the allantoic sac. Goldstein et al. (1979) indicated that cellular proteins and peptides may be transported across cells by receptor mediated endocytosis; however, the presence of Uf receptors in allantoic or chorionic tissues was not examined in this study.

In in vivo studies γ-globulin was introduced into the allantoic fluid of pregnant gilts to determine its uptake by allantoic epithelium and transport into the fetal circulation. Since γ-globulin is not normally found in either allantoic fluid or fetal serum, it served as a convenient probe for these studies. Immunoprecipitable γ-globulin was observed in one-third of the treated fetuses, but none of the control fetuses. The lack of detectable γ-globulin in the remaining treated fetuses using the Ouchterlony double immunodiffusion technique, may be attributed to differences in dilution of the γ-globulin either in allantoic fluid or fetal serum or possibly degradation.

Although uptake of macromolecules by allantoic epithelium does occur, the major portion of ^{59}Fe was in fetal and placental tissues by 24 h after injection of 100 μCi ^{59}Fe into the maternal circulation. These results suggested that the ^{59}Fe-Uf in allantoic fluid may represent only that which was not metabolized by fetal tissues. The fetal vascular drainage from the areolae is into the umbilical veins and almost all of this blood passes through the fetal liver before reaching the heart and being pumped through the arterial system (Patten, 1948). Very little or no umbilical blood is routed through the sinus venosus after about the 12 mm embryo stage of development in pigs. The liver is the primary site of hematopoesis as evidenced by increasing total liver ^{59}Fe ($\bar{X}$ ± SEM) from Day 30 (1809 ± 267 CPM) to Day 60 (56,551 ± 8083 CPM) and Day 90 (72,972 ± 3440 CPM). The erythrocyte forming centers shift to include bone marrow later in gestation as indicated by increasing ^{59}Fe (CPM/g, $\bar{X}$ ± SEM) on Days 60 (466 ± 53), 90 (1146 ± 67) and 105 (1067 ± 67) in the femur.

Further research is underway to determine the precise route of Uf movement from uterine glandular epithelium and into the allantoic fluid and the potential rate of uptake and degradation of Uf by fetal organs.

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REFERENCES

Baker, E. and Morgan, E. H. (1970). Iron transfer across the perfused rabbit placenta. Life Sci. 9:765–772.

Barr, A. J., Goodnight, J. H., Sall, J. P. and Helwig, J. T. (1979). A User's Guide to SAS 79. Sparks Press, Raleigh, NC.

Basha, S.M.M., Bazer, F. W. and Roberts, R. M. (1979). The secretions of a uterine specific, purple phosphatase by cultured explants of porcine endometrium: Dependency upon the state of pregnancy of the donor animal. Biol. Reprod. 20:431–441.

Basha, S. M., Bazer, F. W., Geisert, R. D. and Roberts, R. M. (1980). Progesterone-induced uterine secretions in pigs. Recovery from pseudopregnant and unilaterally pregnant gilts. J. Anim. Sci. 50:113–122.

Bazer, F. W., Chen, T. T., Knight, J. W., Schlosnagle, D. C., Baldwin, N. J. and Roberts, R. M. (1975). Presence of a progesterone-induced uterine specific acid phosphatase in allantoic fluid in gilts. J. Anim. Sci. 41:1112–1119.

Bjorkman, N. H. (1969). Light and electon microscopic studies on alterations in the normal bovine placentome. Anat. Rec. 163:17–29.

Brambell, C. E. (1933). Allantochorionic differentiation of the pig studied morphologically and histochemically. Am. J. Anat. 52:397–457.

Buhi, W. C. (1981). Further characterization and function of uteroferrin from uterine flushings and allantoic fluid of pigs. Ph.D. Dissertation, University of Florida, Gainesville.

Chen, T. T., Bazer, F. W., Cetorelli, J. J., Pollard, W. E. and Roberts, R. M. (1973). Purification and properties of a progesterone-induced basic glycoprotein from the uterine fluids of pigs. J. Biol. Chem. 248:8560–8566.

Chen, T. T., Bazer, F. W., Gebhart, B. M. and Roberts, R. M. (1975). Uterine secretions in mammals: Synthesis and placental transport of a purple acid phosphatase in pigs. Biol. Reprod. 13:304–313.

Clarke, R. M. and Hardy, R. N. (1971). Histological changes in the small intestine of the young pig and their relation to macromolecular uptake. J. Anat. 108:63–77.

Ducsay, C. A. (1980). Uteroferrin: A mechanism of maternal to fetal iron transport in swine. Ph.D. Dissertation, University of Florida, Gainesville.

Faulk, W. P. and Galbraith, G.M.P. (1979). Transferrin and transferrin-receptors of human trophoblast.

In: Protein Transmission Through Living Membranes (W. A. Hemmings, ed.). Elsevier/North Holland Biomedical Press, Amsterdam, pp. 55–61.

Gitlin, D., Kumate, J., Urrusti, J. and Morales, D. (1964). The selectivity of the human placenta in the transfer of plasma proteins from mother to fetus. J. Clin. Invest. 43:1938–1951.

Goldstein, J. L., Anderson, G. W. and Brown, M. S. (1979). Coated pits coated vesicles and receptor mediated endocytosis. Nature 279: 679–684.

Goldstein, M. H., Bazer, F. W. and Barron, D. H. (1980). Characterization of changes in volume, osmolarity and electrolyte composition of porcine fetal fluids during gestation. Biol. Reprod. 22:1168–1180.

Hoskins, F. H. and Hansard, S. L. (1964). Placental transfer of iron in swine as a function of gestation age. Proc. Soc. Exp. Biol. Med. 116:7–11.

Knight, J. W., Bazer, F. W., Thatcher, W. W., Franke, D. E. and Wallace, H. D. (1977). Conceptus development in intact and unilaterally hysterectomized-ovariectomized gilts: Interrelationships among hormonal status, placental development, fetal fluids and fetal growth. J. Anim. Sci. 44:620–637.

Larkin, E. C., Weintraub, L. R. and Crosby, W. H. (1970). Iron transport across rabbit allantoic placenta. Am. J. Physiol. 218:7–11.

Lecce, J. G. (1966). In vitro absorption of γ-globulin by neonatal intestinal epithelium of the pig. J. Physiol. 184:594–604.

Lowry, O. H., Rosenborough, N. J., Farr, A. L. and Randall, R. J. (1951). Protein measurement with the folin phenol reagent. J. Biol. Chem. 193:265–275.

Markwell, M.A.K. (1978). Radioiodination of proteins and peptides using iodo-gen. In: Radioiodination. Pierce Chemical Co. Bulletin. Rockford, IL, pp. 28–30.

Murray, F. A., Bazer, F. W., Wallace, H. D. and Warnick, A. C. (1972). Quantitative and qualitative variation in the secretion of protein by the porcine uterus during the estrous cycle. Biol. Reprod. 7:314–320.

Myagkaya, G. and Vreeling-Sindelarova, H. (1976). Erythrophagocytosis by cells of the trophoblastic epithelium in the sheep placenta in different stages in gestation. Acta Anat. 95:234–248.

Palludan, B. I., Wegger, I. and Moustgaard, J. (1969). Placental transfer of iron. In: Royal Veterinary and Agricultural University Yearbook. Copenhagen, pp. 62–91.

Patten, B. M. (1948). Embryology of the Pig. McGraw-Hill, New York, pp. 227–270.

Roberts, R. M. and Bazer, F. W. (1980). The properties, function and hormonal control of synthesis of uteroferrin, the purple protein of the pig uterus. In: Steroid Induced Proteins (M. Beato, ed.). Elsevier/North Holland, Amsterdam, pp. 133–149.

Schlosnagle, D. C., Bazer, F. W., Tsibris, J.C.M. and Roberts, R. M. (1974). An iron containing phosphatase induced by progesterone in the uterine fluid of pigs. J. Biol. Chem. 249:7574–7579.

Ussing, H. H. and Zerahn, K. (1951). Active transport of sodium as the source of electric current in the short-circuited isolated frog skin. Acta Physiol. Scand. 23:110–127.

Wada, H. G., Hass, P. E. and Sussman, H. H. (1979). Transferrin receptor in human placental brush border membranes. J. Biol. Chem. 254: 12629–12635.

Wislocki, G. B. and Dempsey, E. W. (1946). Histochemical reactions of the placenta of the pig. Am. J. Anat. 78:545–559.

Wohler, F. (1963). The treatment of hemochromatosis with desferrioxamine. Acta Haematol. (Basel) 30:65–87.

Wong, C. T. and Morgan, E. H. (1974). Source of fetal iron in the cat. Aust. J. Exp. Biol. Med. Sci. 52:413–416.