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Synthesis and Release of Polypeptides by Pig Conceptuses During the Period of Blastocyst Elongation and Attachment[1,2]

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ABSTRACT

Pig blastocysts, cultured in a modified minimal essential medium in the presence of radioactive L-leucine, released radiolabeled macromolecules into the medium at a linear rate for at least 24 h. Proteins released into the medium by blastocysts during the period of expansion through attachment were identified by two-dimensional polyacrylamide gel electrophoresis and fluorography. Spherical (4–9 mm), tubular (10–50 mm) and early filamentous blastocysts (>100 mm length), recovered between Days 10.5 and 12 of pregnancy, all released, as their major products, a group of low molecular weight (M_r) acidic polypeptides (M_r 20,000–25,000; pI 5.6–6.2). By Day 13 the pattern of protein synthesis changed markedly and the major proteins detected between Days 13 and 16 were basic and in the molecular weight range of 35,000–50,000. In addition, a high molecular weight glycoprotein of M_r >650,000 was released. The major basic polypeptide (M_r ≃45,000) and the high molecular weight glycoprotein have been isolated from Day 16 blastocyst incubations. After Day 18, a distinctive group of new polypeptides in the 50,000–70,000 molecular weight range were synthesized. These correspond in electrophoretic mobilities to fetal serum proteins, including transferrin, α-fetoprotein and fetuin, and appear to be primarily products of the embryo and/or yolk sac. Explant cultures of Day 25 and 30 chorioallantois cultured without embryonic tissue released three major polypeptides into the medium which were distinct from any synthesized during the preattachment period (Days 10.5–16). Although little is known about the physiological role of conceptus proteins produced early in pregnancy, the identification and isolation of these products is a necessary first step in determining their function.

INTRODUCTION

Establishment of pregnancy in large domestic animals is dependent on complex interactions between the developing conceptus and maternal tissues prior to attachment (implantation). These interactions result in metabolic, endocrinological and immunological adjustments which provide for continued luteal progesterone production, blastocyst nutrition, conceptus growth and immunological privilege. Several products associated with the pregnant uterus and possibly produced by the conceptus have been associated with recognition and maintenance of pregnancy in the large domestic species. These products include steroids (Perry et al., 1973; Bazer and Thatcher, 1977; Flint et al., 1979; Heap et al., 1981), prostaglandins (Henderson et al., 1977; Pratt et al., 1977; Watson and Patek, 1979), and proteins (Cerini et al., 1976; Staples et al., 1976; Martal and Lacroix, 1978; Martal et al., 1979; Findlay et al., 1979; Godkin et al., 1982).

In the pig, there is considerable evidence that estrogens from the blastocyst are responsible for prolonged luteal maintenance and maternal recognition of pregnancy. This state may be achieved through sequestering of prostaglandins within the uterine lumen and endometrium, thus preventing their release into the maternal vasculature (Bazer and Thatcher, 1977). In addition to steroids, the preattachment pig blastocyst is active in syn-

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thesizing and releasing proteins (Wyatt, 1976; Saunders et al., 1980; Rice et al., 1981). Protein extracts from pig conceptuses have been observed to bind to the LH receptor of pig corpora lutea (Saunders et al., 1980). Since proteins synthesized and released by the blastocyst may act as hormones and mediate conceptus-maternal interactions essential for coordinated control of the sequential events of pregnancy, we have attempted to identify and analyze the proteins synthesized by the porcine conceptus during blastocyst elongation (Days 10.5–12) and attachment (approx. Day 18) and the early post-implantation period (Days 25–30). Our approach, which was similar to our study on sheep conceptus proteins (Godkin et al., 1982), was to culture, rather than to extract, blastocysts and then to analyze the secretory proteins released into the incubation medium. The rationale behind these studies was that if blastocyst proteins mediate conceptus-maternal interactions during this period of pregnancy, then these are likely to be among the major proteins released by the conceptus.

MATERIALS AND METHODS

Materials

Tissue culture supplies were purchased from Grand Island Biological Co., Grand Island, NY; acrylamide, N,N,N′, N′ tetramethylenediamine and X-0mat RP film XRP-1 were products of Eastman-Kodak, Rochester, NY, L-[4,5-^{3}H] leucine (sp.act. 62 Ci/mmol) and L-[1-^{14}C] leucine (sp.act. 340 mCi/mmol) were obtained from the Radiochemical Centre, Amersham, England; Coomassie blue R-250 and N,N′ -diallyltartardiamide were from Bio-Rad Labs., Richmond, CA; SDS (specifically pure) and Nonidet P-40 were from BDH Chemicals Ltd., Poole, England; diethylaminoethyl cellulose and carboxymethyl cellulose were purchased from Whatman, Clifton, NJ; ampholines were from LKB, Uppsala, Sweden; amino acids, dithiothreitol, protein standards, 2-mercaptoethanol, agarose, and pregnant mare's serum gonadotropin (PMSG) were from Sigma Chemical Co., St. Louis, MO; urea was from Pierce Chemicals, Rockford, IL; Sephacryl S-200 was a product of Pharmacia Fine Chemicals, Piscataway, NJ. All inorganic chemicals used were reagent grade or better.

Animals

Gilts were observed for estrus in the presence of intact boars daily and the day of the onset of estrus was designated Day 0. Gilts were bred by natural service when detected in estrus, and 12 h and 24 h later. Gilts were hysterectomized under aseptic conditions between Days 10.5 and 18 of pregnancy. Uteri were transferred to a sterile laminar flow hood and conceptuses were flushed from the uterine horns with sterile medium (37°C) and collected in sterile culture dishes. Alternatively, on Days 20, 25 and 30 of pregnancy, gilts were hysterectomized and embryos and placental membranes were dissected out of the uterus aseptically.

Medium Preparation

Eagle's minimum essential medium (MEM) containing phenol red (10 μg/ml) as an indicator, was prepared according to the method of Basha et al. (1979) except that the L- leucine content was limited to 5.2 μg/ml (1/10 normal amount) to enhance the incorporation of the L-[4,5-^{3}H] leucine which was added to the cultures. MEM was supplemented with penicillin (200 units/ml) streptomycin (200 μg/ml) and fungizone (0.5 μg/ml), 0.2 units insulin/ml and 1% (v/v) nonessential amino acids. The medium was filter-sterilized and stored at 4°C until used.

In Vitro Culture of Conceptuses

Conceptuses were removed from the uterus under sterile conditions and transferred to sterile Petri dishes containing 15 ml MEM and either 100 μCi L-[^{3}H] leucine or 50 μCi L-[^{14}C] leucine. Each dish was then transferred to a controlled atmosphere chamber (Bellco Biological Glassware, Vineland, NJ). The chamber was flushed for 1 min with 50% O_2-45% N_2-5% CO_2 (by volume) and incubated at 37°C in the dark on a rocking platform (6 cycles/min) according to the method of Basha et al. (1979). This ensured that the cultures were alternately immersed in medium and then exposed directly to the gaseous atmosphere. The technique, originally described by Barrett et al. (1976), is now used widely for culture of tissue and organ explants from a variety of sources (see Trump et al., 1980) and has been employed to maintain viable uterine endometrial explants from pigs for up to 50 days (J. D. Godkin and R. M. Roberts, unpublished observation) and sheep conceptus cultures for 48 h (Godkin et al., 1982). After 24–48 h, incubations were terminated by chilling the incubation mixture on ice and then centrifuging at 12,000 × *g* for 20 min to separate tissue and medium. The medium was dialyzed extensively (molecular weight cut off ≃3500) against 10 mM Tris-HCl buffer (pH 8.2) to remove low molecular weight compounds, e.g., salts and unincorporated radiolabeled precursors.

Embryos within the amnion were collected by gently teasing open the chorion (Day 18) or chorioallantois (after Day 20) and dissecting the embryo-amnion away from these membranes. The embryo and various tissues were then isolated and cultured as described above.

Synthesis and Release of Protein

Proteins synthesized and released into the incubation medium were monitored by observing the incorporation of radiolabeled amino acids into macromolecules over a 24-h incubation period. Aliquots (0.4 ml) of medium were removed at regular intervals, dialyzed extensively against 10 mM Tris-HCl (pH 8.2) and radioactive content measured to determine the time course of appearance of nondiffusible material (presumably proteins and complex saccharides).

Preparation of Samples for Electrophoresis

Dialyzed medium (3 ml) was lyophilized and the dried material dissolved in 1 ml 5 mM K_2CO_3 containing 9.3 M urea, 2% (v/v) Nonidet P-40 and 0.5% (w/v) dithiothreitol and used for two-dimensional polyacrylamide gel electrophoresis (2D-PAGE). Conceptus tissue was suspended in 0.5 ml of 5 mM K_2CO_3 and 9.3 M urea and sonicated (4 × 15 sec at 1 min intervals) in an ice bath using a Bronwill Biosonic at 20–30% probe intensity. The sonicated material was made to 2% (v/v) with Nonidet P-40 and 0.5% (w/v) with dithiothreitol and centrifuged at 20,000 × *g* for 30 min at 18°C. The clear supernatant solution was used for electrophoretic analysis of polypeptides.

Two-dimensional Polyacrylamide Gel Electrophoresis (2D-PAGE)

This analysis was performed according to a modification of the method of O'Farrell (1975) described by Horst and Roberts (1979), Horst et al. (1980) and as employed by Godkin et al. (1982) for analysis of proteins released into the medium by cultures of ovine conceptuses.

Isoelectric Focusing

Proteins of the tissue and medium were subjected to isoelectric focusing in the first dimension in 4% (w/v) acrylamide gels containing N,N′ diallyltartardiamide (as a cross-linker), 9.3 M urea, 2% (v/v) Nonidet P-40 and 2% ampholines (3.5–5, 5–7 and 9–11, 50:35:15 by volume, respectively). The gels were then equilibrated for 10 min in 0.065 M-TrisHCl buffer (pH 6.9) containing 1% (w/v) SDS and 1% (v/v) 2-mercaptoethanol.

Second Dimension Electrophoresis

SDS-gel electrophoresis was performed according to the method of Laemmli (1970). The equilibrated tube gels, after electrofocusing, were overlayed on 10% (w/v) acrylamide slabs and electrophoresis performed toward the anode. The slabs were fixed and stained with Coomassie blue R-250. Slab gels were impregnated with sodium salicylate (1 M; 30 min) (Chamberlain, 1979), dried and fluorographs prepared (Laskey and Mills, 1975) from Kodak XRP-1 X-ray film.

Protein Fractionation by Ion-Exchange and Gel Filtration Chromatography

Nondiffusible material retained after dialysis of incubation medium was adsorbed onto a diethylaminoethyl cellulose (DEAE) column (1.5 × 10 cm) equilibrated with 10 mM Tris-HCl (pH 8.2) or a carboxymethyl cellulose column (CM-cellulose) (1.5 × 5 cm) equilibrated with 10 mM sodium acetate (pH 5.4). After washing the column thoroughly, the bound protein was eluted with 300 ml of a linear salt gradient (0–0.5 M NaCl) and 3 ml fractions were collected. The radioactive content of each fraction was determined and protein peaks were pooled and dialyzed extensively against 10 mM Tris-HCl (pH 8.2) or 10 mM sodium acetate buffer (pH 5.4). Aliquots of dialyzed protein peaks from ion-exchange chromatography were lyophilized and submitted to two-dimensional or single-dimensional (Laemmli, 1970) polyacrylamide gel electrophoresis, enabling visual identification of the polypeptides composing each peak. Dialyzed proteins of peaks were also concentrated by exchanging the material onto a short (1 × 0.5 cm) DEAE-cellulose or CM-cellulose column and eluting the bound material with 3–5 ml of 0.5 M NaCl. Concentrated samples were then submitted to gel filtration chromatography on a Sephacryl S-200 column (90 × 1.5 cm) or a Sepharose 6B column (90 × 1.5 cm) equilibrated in 10 mM Tris HCl, 0.3 M NaCl (pH 8.2). Protein standards used with Sephacryl S-200 were blue dextran, bovine serum albumin, ovalbumin, chymotrypsinogen and ribonuclease A. Blue dextran, thyroglobulin and bovine serum albumin were employed with Sepharose 6B columns.

Protein Determination

Protein concentrations were determined by the method of Lowry et al. (1951) using bovine serum albumin as the standard.

RESULTS

Release of Radioactivity into the Medium

Porcine blastocysts were incubated at different stages of development in medium in the presence of radioactive L- leucine to follow the release of proteins synthesized by tissues during incubation. Since there are very rapid changes in blastocyst morphology between Days 10 and 13 of pregnancy (Anderson, 1978) and because there is considerable variation between animals in these early stages of elongation, blastocysts were classified according to their individual stage of development (i.e., spherical, 5–10 mm diameter; tubular, 11–50 mm; and filamentous, >100 mm. Once the blastocysts have elongated beyond about 10 cm their exact length is impossible to determine since they become inextricably tangled when flushed from the uterus. Such blastocysts were identified as filamentous. At all stages of pregnancy tested, conceptus tissues released considerable amounts of macromolecular material (presumbaly proteins) into the medium. However, a detailed time course, employing several conceptuses has only been carried out with Day 16 material (Fig. 1). Here, release was approximately linear between 4 and 24 h. A slight lag was evident during the initial 4 h, but there was no evidence for a decrease in the synthetic activity towards the end of the experiment which might have indicated loss of tissue viability.

Analysis of Polypeptides by 2D-PAGE

Analysis of polypeptides present in the incubation medium and in tissue from con-

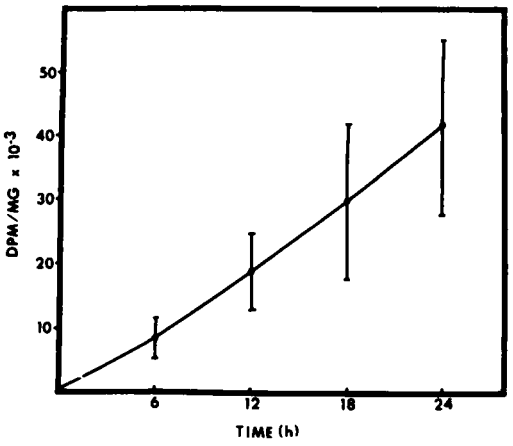

FIG. 1. Release of ^{14}C-labeled, macromolecular components by Day 16 conceptuses over a 24-h period. Conceptuses from each uterine horn were incubated in 15 ml of medium containing 50 μCi of L-[^{14}C] leucine. Samples of medium were withdrawn at 6-h intervals, dialyzed against 10 mM Tris-HCl buffer (pH 8.2) and their radioactivity measured. Values are expressed as dpm/mg wet tissue weight. Results are for 5 experiments ($\bar{X}$ ± SEM).

ceptuses removed from the uterus of gilts on Days 10.5 through 30 were carried out by 2D-PAGE, followed by fluorography of the dried gels. During the initial stages of blastocyst elongation (spherical, tubular and early filamentous; Days 10.5–12) the polypeptides synthesized and released into the medium in major amounts constituted a group of low molecular weight acidic proteins. As seen in Fig. 2a, these proteins had a molecular weight of about 20–25,000 as determined by their electrophoretic mobilities in the presence of SDS. The isoelectric points (pI) under denaturing conditions in the presence of urea were between 5.6 and 6.2. By Day 13 of pregnancy the pattern of protein synthesis by filamentous blastocysts became more complex and, although the low molecular weight acidic proteins were still present, the dominant polypeptides were basic and of higher molecular weight (Fig. 2b). Between Days 13 and 16 this pattern of protein synthesis and release remained relatively unchanged (Fig. 2c) and the major products continued to be basic proteins with molecular weights of about 40–50,000. At Day 18 the basic and low molecular weight proteins were still present, but diminished in proportion, while new products of higher molecular weight appeared (Fig. 2d). We demonstrated that these higher molecular weight proteins were primarily embryonic products by dissecting the embryo and amnion away from the trophoblast (chorioallantois) and culturing them separately (Fig. 4). At Day 20, protein synthesis by the trophoblast continued to decline while polypeptides assumed to be produced by embryonic tissues became more evident. At Days 25 and 30 explant cultures of chorioallantois released no detectable basic proteins in the 40–50,000 molecular weight range into the medium, demonstrating the declining protein secretory activity of these tissues. In addition, the low molecular weight acidic proteins seen between Days 10.5 and 18 were undetectable. Proteins which were present were two relatively low molecular weight acidic proteins and a protein(s) in the 50–60,000 M_r range which appeared as a narrow band spanning pI's of 6.0–7.3. None of these three products were detected on fluorographs produced from cultures of conceptuses between Days 10.5–16.

Analysis of tissue proteins synthesized during the incubation showed a pattern of labeled polypeptides distinct from that in the medium for blastocysts at all stages of elongation. In Fig. 3, for example, analysis of labeled tissue at Day 16 (*b*) and at an earlier, spherical stage (*a*) are shown. The major proteins on the gels were in the M_r range of 35,000–60,000. Little of the low molecular weight acidic polypeptides released by early blastocysts or of the basic protein species released by filamentous blastocysts could be detected.

On Days 18, 20 and 25, embryos within their amnion were dissected out of the chorioallantois and the embryo-amnion units were cultured separately from the chorioallantois. 2D-PAGE analysis of medium from these cultures followed by fluorography demonstrated that embryos at these stages of development synthesize and release several major polypeptides in the 50–70,000 M_r range (Fig. 4). We have compared, by 2D-PAGE analysis, the protein profiles of these studies with those generated from protein analysis of porcine fetal plasma obtained at Day 60 of pregnancy (Buhi et al., 1982) The major proteins in the 50–70,000 M_r range from the present study correspond identically in pI's and electrophoretic mobilities with the major fetal plasma proteins (Buhi et al., 1982). For example, in Fig. 4b, each polypeptide labeled *a-f* corresponds in molecular weight and pI to one of the major six polypeptides observed on 2D-PAGE maps from Day 60 fetal pig plasma (see Buhi et al., 1982).

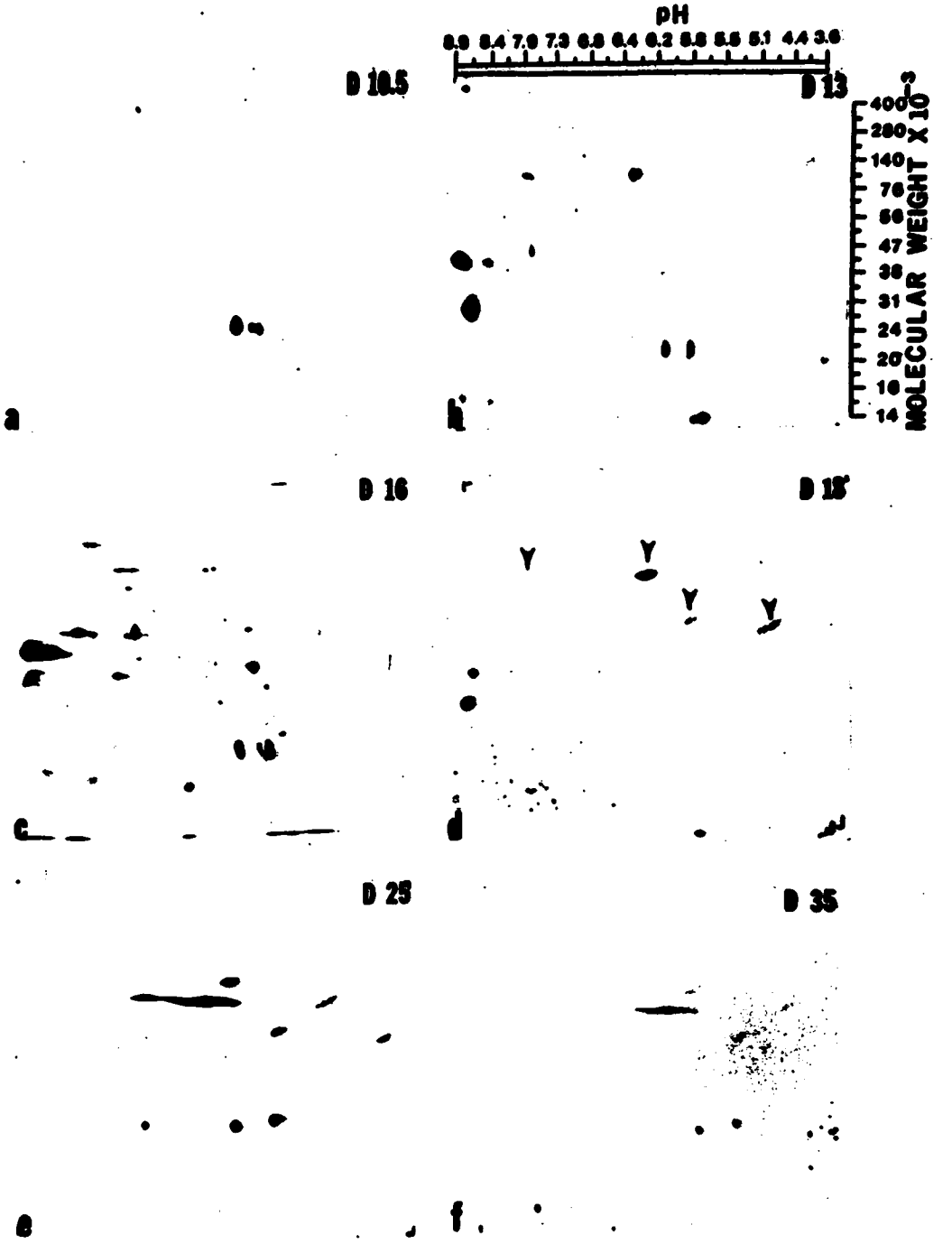

FIG. 2. Fluorographs of dried, two-dimensional polyacrylamide gels for analysis of radioisotopically labeled proteins released into the incubation by conceptuses following culture with L-[^{3}H] leucine. *a*) Medium from a Day 10.5, 4 mm spherical conceptus culture (20,000 cpm L-[^{3}H] leucine-labeled protein loaded on gel; fluorograph exposed for 10 days). *b*) Medium from a Day 13 conceptus culture (232,000 cpm L-[^{3}H] leucine-labeled protein loaded on gel; fluorograph exposed for 7 days). *c*) Medium from a Day 16 conceptus culture (190,000 cpm L-[^{3}H] leucine-labeled protein loaded on gel; fluorograph exposed for 7 days). *d*) Medium from a Day 18 conceptus culture (141,000 cpm L-[^{3}H] leucine labeled protein loaded on gel; fluorograph exposed for 7 days). *Arrows* indicate serum-like proteins synthesized by the cultures at this stage of pregnancy. *e*) Medium from a Day 25 chorioallantois culture (82,000 cpm L-[^{3}H] leucine-labeled protein loaded on gel; fluorograph exposed for 21 days). *f*) Medium from a Day 35 chorioallantois culture (60,000 cpm L-[^{3}H] leucine-labeled protein loaded on gel; fluorograph exposed for 21 days).

FIG. 3. Fluorograph of two-dimensional polyacrylamide gels in which solubilized whole tissue from a spherical (9 mm) blastocyst (*a*) and a Day 16 conceptus (*b*) was analyzed. Note the absence of the proteins which appeared in the incubation medium in Figs. 2a and 2c.

Ion Exchange Chromatography

Dialyzed incubation medium from Day 16 blastocyst incubations were first submitted to DEAE ion exchange chromatography. Acidic proteins were bound by the gel while basic proteins passed through. The acidic proteins were then eluted with a linear NaCl gradient resulting in the elution pattern shown in Fig. 5. Peak 1 (10–50 ml) constitutes the basic proteins. Peak 2 (≃100–120 ml) which elutes early in the gradient (approx. 0.05 M NaCl) has been demonstrated to be a glycoprotein of very high molecular weight (Godkin et al., 1981). When we attempted to analyze this material by single dimension electrophoresis, after boiling in SDS and 2-metcaptoethanol, the radioactivity was recovered either in the stacking gel or at the very top of the running gel. Attempts to analyze this material by 2D-PAGE have been unsuccessful since it fails to enter the first dimension isoelectric focusing gel. Polypeptides in DEAE peak 2 (100–120 ml) do not, therefore, appear on the gels shown in Fig. 2. When this material was concentrated and submitted to gel filtration it was found to elute at the void volume of the Sephacryl S-200 column and ahead of thyroglobulin dimer on the Sepharose 6B column (results not shown). Its molecular weight was, therefore, estimated to be greater than 660,000.

When labeled materials which eluted later in the gradient were concentrated and submitted to 2D-PAGE analysis (results not shown), a variety of individual polypeptides were detectable but none of these appeared to be produced in major amounts.

Proteins which did not bind DEAE-cellulose were dialyzed against 10 mM sodium acetate buffer (pH 5.4) and submitted to CM-cellulose ion-exchange chromatography (Fig. 6). The radioactivity in this material eluted as three distinct peaks during the NaCl gradient. Each of these peaks were concentrated and submitted to 1D PAGE analysis (Fig. 8b). Peak I was composed of three protein bands of low molecular weight (≃14–20,000). Peak II was composed primarily of two bands with the major component having a molecular weight of about 50,000 and the other about 25,000. CM-cellulose peak III was composed primarily of a protein with a molecular weight of about 45,000. This protein was the major single protein synthesized by Day 14–16 blastocysts. The CM-cellulose peak III was concentrated and chromatographed on a Sephacryl S-200 column where it eluted as a single symmetrical peak of radioactivity (Fig. 7). Fluorography of dried two-dimensional gels of this material revealed only one spot indicating the protein to be radiochemically pure (Fig. 8a).

DISCUSSION

Between Days 10.5 and 12 of pregnancy the pig blastocyst undergoes a dramatic morphological reorganization from a small sphere about 4 mm in diameter to a narrow filamentous structure about 1 m in length and 0.75 mm in diameter (Perry and Rowlands, 1962). About Day 11, when the blastocysts are beginning to elongate and are about 10 mm in length, they start to acquire steroidogenic capabilities (Gadsby and Heap, 1978) and to signal their

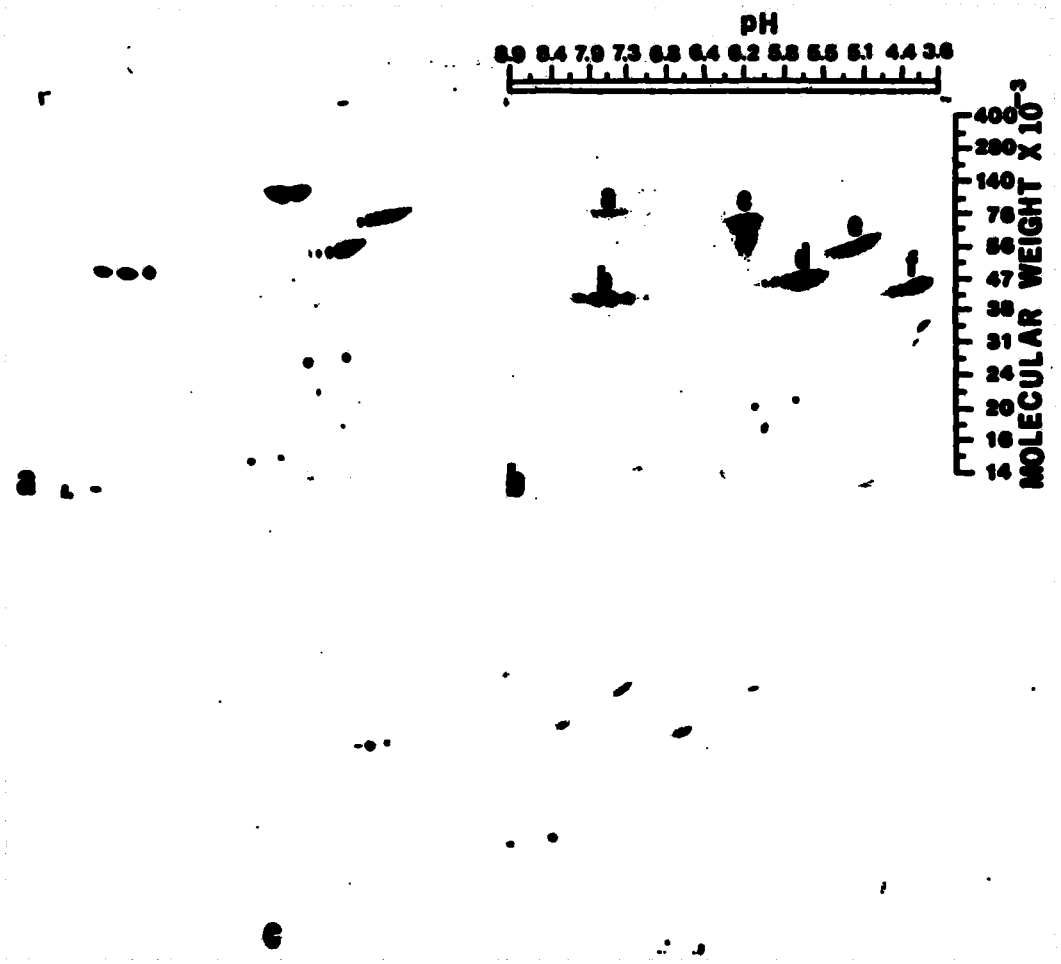

FIG. 4. Fluorograph of two-dimensional polyacrylamide gels from cultures of embryos plus their amnions which had been dissected away intact from the chorioallantois. *a*). Medium from Day 18 culture (220,000 cpm L-[^{3}H] leucine-labeled protein loaded on gel; fluorograph exposed for 7 days). *b*) Medium from Day 20 culture (200,000 cpm L-[^{3}H] leucine-labeled protein loaded on gel; fluorograph exposed for 7 days). *c*) Medium from Day 25 culture (82,500 cpm L-[^{3}H] leucine-labeled protein loaded on gel; fluorograph exposed for 7 days). In gel *b* the letters above the polypeptides refer to those products which have been tentatively identified by their electrophoretic mobilities (Anderson and Anderson, 1977) and similarities to fetal porcine plasma proteins as: *a*, transferrin; *b*, fibrinogen β-chain; *c*, α-fetoprotein; *d*, α_1 antitrypsin; *e*, α_1 antichymotrypsin; and *f*, fetuin.

presence to maternal tissues (Dhindsa and Dzuik, 1968). As shown here, blastocysts in the spherical, tubular and early filamentous stages (Days 10.5 to 12) also release a group of low molecular weight acidic proteins when cultured in vitro.

Shortly after reaching the filamentous stage (Days 12–13), the pattern of protein synthesis changed. Although the low molecular weight proteins persisted, the polypeptides produced in major amounts were basic and in the 40–50,000 M_r range. All these products were released selectively into the medium and were probably secreted, and not merely leached from the blastocyst, as evidenced by the absence of significant amounts of tissue proteins in the medium. It is likely, therefore, that they are also released by unattached blastocysts when in utero.

It is interesting that the expanding sheep blastocyst also produces a group of low molecular weight, acidic proteins (Godkin et al., 1982) which are similar in their physical properties to the ones described here for the pig. In the sheep these products are released from the trophoblast transiently between Days

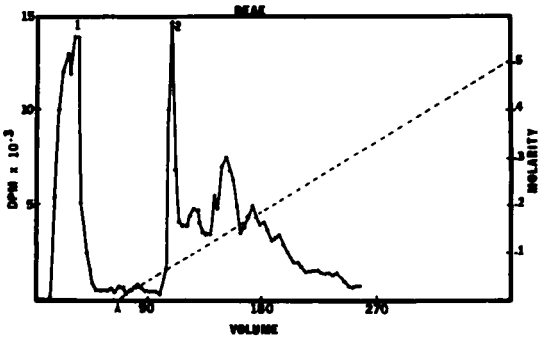

FIG. 5. Diethylaminoethyl (DEAE) cellulose ion-exchange chromatography of dialyzed medium from incubation of Day 16 conceptuses with 100 μCi of L-[^{3}H] leucine added. *Arrow* indicates start of NaCl gradient (shown by *broken line*). Peak 1 is composed of basic proteins which did not bind to the gel. Peak 2 is composed of the high molecular weight glycoprotein referred to in the text.

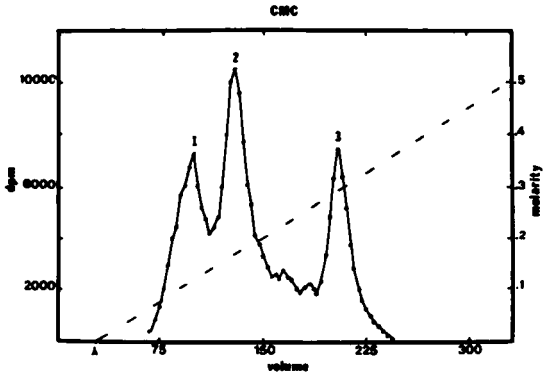

FIG. 6. Carboxymethyl (CM)-cellulose ion-exchange chromatography of protein which did not bind to DEAE-cellulose (Fig. 5) was loaded onto a CM-cellulose column and eluted with a gradient of NaCl. *Arrow* indicates start of gradient; *broken line* shows the concentration of NaCl. Peaks 1, 2 and 3 were collected for further analysis.

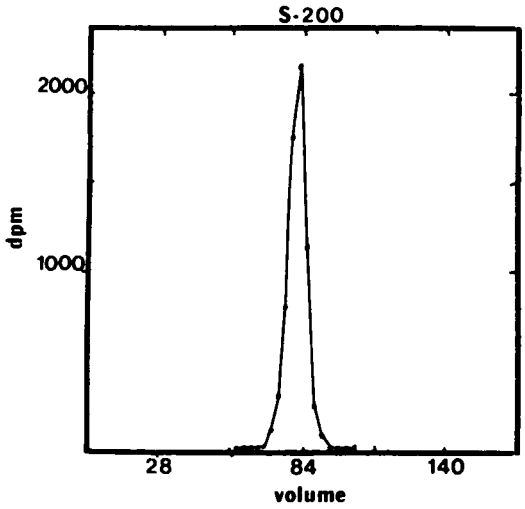

FIG. 7. Sephacryl S-200 gel filtration chromatography of peak 3 from CM-cellulose ion-exchange chromatography. Material from peak 3 (Fig. 6) was concentrated and subjected to chromatography as described in *Materials and Methods*.

13 and 21 at a time coinciding with the maternal recognition of pregnancy. As demonstrated by immunocytochemical techniques, the proteins become associated with the uterine epithelium of the pregnant ewe (Godkin et al., 1981). Possibly these proteins, which appear to be the first proteinaceous products released in major amount by the pig and sheep blastocysts, have similar functions in the two species.

Another similarity between the pig and sheep at these early stages of pregnancy is that the expanding blastocysts of both species produce a product of high molecular weight which elutes early in the NaCl gradient during DEAE-ion exchange chromatography (Godkin et al., 1981). In both species this compound had a molecular weight greater than 600,000 and a carbohydrate content of roughly 50%. This carbohydrate was made up of N- rather than O-linked chains and was composed largely of D-galactose and N-acetyl D-glucosamine residues (Godkin et al., 1981). Since an identical type of glycoprotein was also released by the Day 16 cow blastocyst, it again seems likely that the protein may serve a common function in these large domestic species.

Proteins resembling the basic components released by the Day 13–18 pig conceptus are not formed in the sheep (see Godkin et al., 1982). However, our ability to purify one of these basic proteins by a combination of ion exchange and gel chromatography might now allow us to test its function in the pig without interference from a complex mixture of other proteins and blastocysts metabolites.

The allantoic membrane develops rapidly after Day 16 of pregnancy and fuses with a small portion of the chorion on about Day 20. By Day 24 the fusion of the chorion and allantois is completed, forming the chorio-allantoic membrane (Heusen, 1927; Amoroso, 1952). In order to clarify the terminology we shall refer to the outer extraembryonic mem-

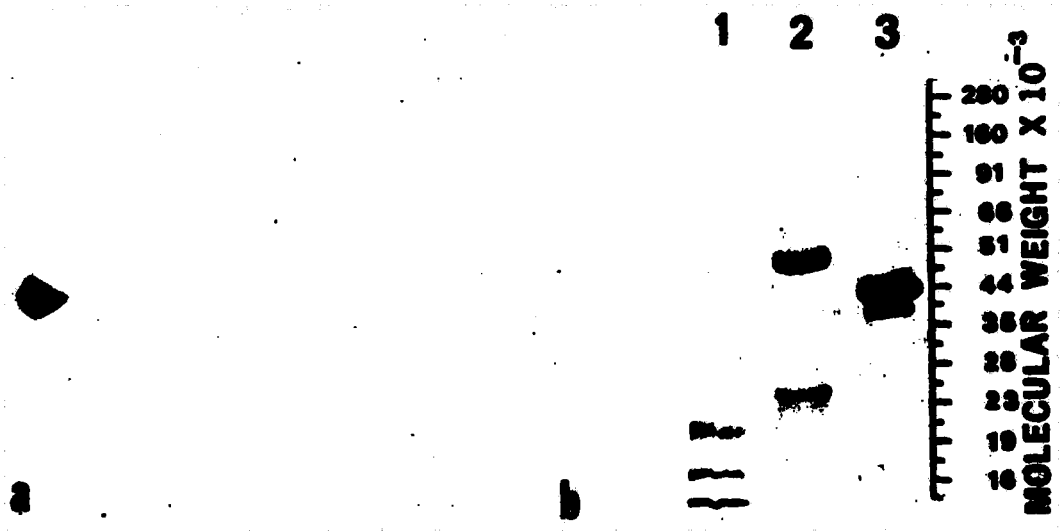

FIG. 8. Polyacrylamide gel electrophoresis of polypeptide components fractionated by CM-cellulose ion-exchange and Sephacryl S-200 gel chromatography. In *b*, lane 1 contains material from CM-cellulose peak 1, lane 2 from peak 2 and lane 3 from peak 3. Gels were stained with Coomassie blue. *a*) Fluorograph of two-dimensional polyacrylamide gel of CM cellulose peak 3 following Sephacryl S-200 gel filtration.

brane as the trophoblast up to Day 16. Between Days 18 and 20 we shall refer to this membrane as the chorion and after Day 20 we will call it the chorioallantois.

By Day 18 the release of labeled protein appeared to decline. This time period coincides with attachment of the chorion to the maternal uterine endometrium by interdigitation of microvilli (see Perry, 1981). By Day 25, proteins synthesized and released by explants of chorioallantois were very different from those produced by conceptus incubations between Days 13–18. Day 25 chorioallantois, for example, produced no detectable basic proteins in the 40–50,000 M_r range. The major proteinaceous products were of relatively low molecular weight (approx. 30,000), plus a product in the 50–60,000 M_r range which appeared on fluorographs as a narrow streak spanning the isoelectric point range 7.3–6.0.

At Day 18, the embryo contributes to the proteins released by the conceptus in culture. We have demonstrated this by dissecting the embryo (contained in its amnion) out of the chorion or choriallantois and incubating the embryo-amnion unit separately, and then comparing the 2D-PAGE gels of the incubation medium with those produced from whole conceptus incubations. The proteins synthesized in major amounts by the embryo between Days 18–25 were a group with varied pI's and in the 50–70,000 M_r range.

Although we have not positively identified these six major proteins (Fig. 4b, *a-f*) produced by the embryo cultures as plasma proteins, we have demonstrated that they have the same electrophoretic characteristics as the six major proteins which we have observed in porcine fetal plasma. Others (Anderson and Anderson, 1977) have identified human plasma proteins by sensitive immunoprecipitation techniques and produced a 2D-PAGE "map" of these proteins. Several of the embryo culture proteins and fetal plasma proteins from our pig studies corresponded in electrophoretic mobilities with human plasma proteins.

Fetuin, α-fetoprotein and transferrin have been detected in the plasma and cerebral spinal fluid of fetal pigs at Days 45 through 105 (Dziegiezewska et al., 1980). The synthesis and release of similar proteins by embryos was detected as early as Day 18 in the present study. Both yolk sac and liver have been implicated in the production of such "serum" proteins in several species (for review of neonatal and fetal plasma proteins see Gitlin and Gitlin, 1975).

The yolk sac of the pig conceptus starts to develop around Day 14 and reaches its maximum development around Day 18 (Perry, 1981). It then progressively shrinks in size, becoming an inconspicious structure by Day 40

(Tiedeman and Munth, 1980). It is possible that the embryo cultures at Days 18–25 were contaminated with yolk sac tissue which contributed (along with the fetal liver) to the production of "serum-like" proteins. In addition, it is also likely that the "serum-like" proteins observed in the medium of chorion and chorioallantois cultures resulted from contamination by yolk sac tissues and were not products of trophectoderm.

In conclusion, therefore, we have demonstrated that the patterns of proteins released by the developing pig conceptus change markedly during the early stages of pregnancy, even prior to attachment. Some of these products are similar to ones detected in other domestic species. At this time we know little about the function of these proteins, but their identification and purification are necessary first steps in attempts to understand their role during early pregnancy.

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