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H. G. Kattesh, Major Professor

We have read this thesis and recommend its acceptance:

J. D. Godkin, T. T. Chen

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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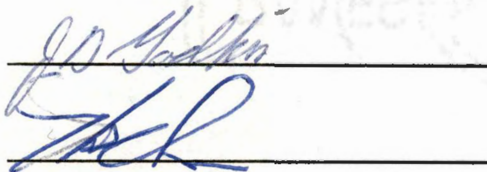

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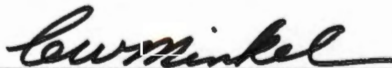

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Date November 1986

DEVELOPMENT OF ANTISERUM AGAINST THE MAJOR BASIC PROTEIN ISOLATED FROM THE MEDIUM OF PERI-ATTACHMENT PORCINE CONCEPTUS CULTURES

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Allison Henson Climer

December 1986

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Thesis
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I dedicate this manuscript to a man who had a great respect for knowledge, wisdom and truth and who taught me to have pride and confidence along with numerous other values and skills. I dedicate this thesis to my loving father, Christle Henson.

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ABSTRACT

Studies were performed to isolate the major secretory protein produced by the peri-attachment porcine conceptus and produce antiserum against it. Gilts were surgically hysterectomized on day 16 +/- 1 post-breeding and, conceptuses flushed from uteri and cultured for 24 hours in a minimum essential medium with either ^{3}H-leucine or ^{35}S-methionine. Proteins released into the incubation medium were subjected to carboxymethylcellulose ion exchange and Sephacryl-200 gel filtration chromatography to isolate the protein. Isolation of the protein (Mr 43000; pI 7.5-8.0) from other components of conceptus secretion was verified by non-equilibrium pH gradient electrophoresis and fluorography.

A rabbit received an initial injection of 720 ug of the isolated protein in Freund's complete adjuvant and was boosted with 160 ug protein in Freund's incomplete adjuvant four weeks later. Using Ouchterlony immunodiffusion, antibody against the isolated conceptus protein was identified in the serum of the treated rabbit. Antiserum was characterized by Ouchterlony immunodiffusion, antigen-antibody immunoprecipitation, one dimensional polyacrylamide gel electrophoresis and fluorography. The molecular weights of the labelled Ag-Ab complex and the peri-attachment conceptus protein in major production are approximately 43000. The antiserum did not crossreact with 1) uterine flushings from pseudopregnant gilts, 2) peripheral plasma from nonpregnant gilts, or 3) amnionic and allantoic fluid and media from cultures of endometrial, chorionic or amnionic tissue from late pregnant gilts. This antiserum will be used in

future studies designed to examine the site of action and function of the major protein product of the peri-attachment porcine conceptus.

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CRANE'S CREST

CHAPTER 1

INTRODUCTION

Embryonic loss in the pre- and peri-attachment periods is typically high in swine. By day 17 of pregnancy, the embryonic mortality rate averages approximately 17% and this rate increases to approximately 33% by day 25 (Flint et al., 1982). It is during this time of conceptus attachment, when the blastocyst is exchanging uterine secretions for a placental supply of nutrients, that the embryonic mortality rate is greatest. Steroid and protein exchanges between conceptus and endometrium could well play a role in regulating transfer from one source of nutrition to another.

The conceptus can derive nutrients either from circulating maternal blood (haemotroph) or from cell debris, uterine secretions and breakdown products of the endometrium and conceptus tissues (histotroph). Swine conceptuses undergo a non-invasive prolonged epitheliochorial attachment that is not complete until day 18 of gestation. Therefore, until day 18 the conceptus is dependent upon histotroph which is absorbed by columnar trophoblast (Dantzer et al., 1981). Results of immunofluorescence studies support the concept that macromolecular components of histotroph are secreted by surface and glandular epithelium of the endometrium and pass into the fetus via chorioallantoic areolae which develop opposite maternal endometrial glands (Chen et al., 1975a,b; Friess et al., 1981). Also, histotroph is intermediary for conceptus waste removal. Before the period of maternal recognition of pregnancy (day 12), histotroph is controlled

by ovarian hormones (Cook and Hunter, 1978). After day 12 and throughout the attachment process, estrogen produced by the rapidly expanding blastocyst increases secretion from the uterine endometrium (Geisert et al., 1982c). Steroids, proteins, prostaglandins and other compounds exchanged between the conceptus and uterus could trigger and/or regulate other events of early pregnancy. Further studies are needed to characterize specific conceptus secretions and to determine their actions during early pregnancy. A better understanding of the contributions made by the conceptus may lead to ways to reduce early embryonic mortality. The objectives of this study were to isolate the major basic protein produced by the peri-attachment porcine conceptus and develop an antiserum against it.

CHAPTER 2

REVIEW OF LITERATURE

Early Conceptus Development (Days 1-21)

In swine estrus lasts 24-72 hours and recurs in 18-21 day cycles. Spontaneous ovulation, which occurs approximately 36 hours following the onset of estrus (day 0), results from a surge of luteinizing hormone (LH; Perry and Rowlands, 1962; Austin, 1969). Fertilization occurs in the ampulla portion of the oviduct approximately 6-12 hours post coitus (Austin, 1969). Twenty to 50 hours post coitus in swine, fertilized eggs undergo the first cleavage (Austin, 1969). Cleavage results in the production of many smaller cells called blastomeres that are necessary for differentiation to occur. This solid mass of cells is referred to as the morula (Perry and Rowlands, 1962; Noden and de Lahunter, 1985). The first event of embryogenesis is the polarization of individual blastomeres into trophoblast and inner cell mass. The blastocyst is formed at the late morula stage when fluid passes into the intercellular spaces between the inner and outer layers of blastomeres. This forms a hollow ball of trophoblast cells with a fluid filled cavity and the inner cell mass which develops into the embryo (Austin, 1969).

Approximately 60-72 hours after the onset of estrus, fertilized porcine ova (zygotes) at about the 4 cell stage enter the uterus (Bazer and First, 1983). Murray and coworkers (1971) restricted the oviduct by ligations at the uterotubal junction and found that if the embryo fails to leave the oviduct, development stops at the late

morula or early blastocyst stage which is usually reached by day 5 or 6 (Austin, 1969; Marrable, 1971; Pope and Day, 1972; Bazer and Roberts, 1983). An in vitro study shows that estradiol-17B in uterine fluid is an essential factor for the transformation of the morula to the blastocyst (Elsaesser and Niemann, 1985).

Porcine blastocysts remain near the tip of the horn until about day 6 when they begin to migrate towards the body of the uterus (Dzuik, 1985). Between days 6 and 8 when the spherical blastocyst is approximately 2 mm in diameter, the zona pellucida (inner egg membrane) is shed and the process is termed hatching (Perry and Rowlands, 1962; Perry et al., 1976; Gadsby and Heap, 1978; Johnson and Ulberg, 1985). After day 7, the trophectoderm cells develop a dense population of microvilli on the apical surface that will be implemented in endocytosis of histotroph and intrauterine migration (Geisert et al., 1982b; Bazer and Roberts, 1983; Johnson and Ulberg, 1985). The day 9 blastocyst is still spherical but the diameter has increased three times since hatching and the number of trophoblast cells has increased to several thousand (Anderson, 1978). By day 9, some blastocysts have entered the horn opposite the one of its origin and continue migration until day 15 where they remain approximately equidistant (Cook and Hunter, 1978; Dzuik, 1985) and form a distinct pattern by days 11-13 (Anderson, 1978; Cook and Hunter, 1978). By day 11, the inner cell mass has differentiated into the embryonic disc and is covered externally with microvilli which stop at the interface of the disc and trophoblast (Johnson and Ulberg, 1985).

The time of maternal recognition of pregnancy (days 10-12) is the period before attachment of the trophoblast (outer extra-embryonic membrane) to the uterine wall (Dhindsa and Dzuik, 1968; Bazer and Thatcher, 1977; Geisert et al., 1982b). Maternal recognition of pregnancy occurs when conceptus-maternal interactions result in maintenance of corpora lutea (CL) and continued production of progesterone (P_4; Bazer and First, 1983). Embryos must be present in both horns by days 10-12 for continuation of pregnancy since regression of the CL on days 14-16 is bilateral. After day 12, unilateral pregnancy will continue (Dhindsa and Dzuik, 1966; Dhindsa and Dzuik, 1968; Moor, 1968). At least 4 blastocysts must be present in the porcine uterus during the second week of pregnancy in order to prevent CL regression (Polge et al., 1966). This latter study found that if only one blastocyst is present, the cycle length may be extended by about 6 days.

Between days 10-16, the blastocyst undergoes a period of rapid expansion along with an increase in DNA and RNA reflecting high mitotic activity (Geisert et al., 1982b). The blastocyst expands from a 0.5-1.0 mm spherical form to 3-10 mm in diameter by day 10 through cellular hyperplasia (Anderson, 1978; Geisert et al., 1982b). The day 11 blastocyst is tubular and approximately 58 mm long (Anderson, 1978). The rapid elongation at a rate of 30-45 mm/sec to the 100-200 mm filamentous form by days 12-14 is a result of cellular remodelling (Geisert et al., 1982b). Elongation provides greater blastocyst-uterine luminal surface contact thus insuring the transmission of signals for establishment of pregnancy (Perry and Rowlands, 1962).

Continued elongation and growth of the blastocyst from 100-200 mm to 800-1000 mm length between days 14-16 involves cellular hyperplasia (Anderson, 1978; Geisert et al., 1982b).

Events associated with blastocyst elongaton (days 10-12) leads to secretion and sequestering of histotroph within the uterine lumen for utilization by the developing conceptus (blastocyst and its extra-embryonic membranes; Zavy et al., 1980; Geisert et al., 1982a). Before attachment, the conceptus is dependent on the secretions of the oviducts and uterine mucosa and the yolk sac (which develops on days 13-16; Marrable, 1971; Perry, 1981) for nutrition and development particularly in animals where blastocyst expansion and development precedes implantation or attachment (e.g. cow, sheep, pig; Austin, 1969; Sauer, 1979). In swine, days 13-14 is the time period for the loose initial attachment of the trophoblast to the uterine epithelium (Geisert et al., 1982b). There is no invasion of maternal uterine endometrium by blastocysts. Therefore, the term "placentation" or "attachment" is more appropriate than "implantation" (Perry et al., 1976; Gadsby and Heap, 1978; Bazer and First, 1983). When conceptuses were transferred to spleen, kidney and ureter tissues, ovarian stroma, uterine mucosa and the outer wall and between the muscle layers of the uterus, their attachment was invasive (Samuel, 1971; Samuel and Perry, 1972). This form of implantation with cytolytic and phagocytic properties is not found in the normal situation, suggesting that its normal development is regulated by the uterine environment.

By day 18, the attachment between fetal trophoblast cells and maternal epithelia by interlocking microvilli, forming the epithelial

chorial placenta (diffuse), is complete and, fetal-maternal exchange of gases and nutrients is established (Perry et al., 1973; Gadsby and Heap, 1978; Perry, 1981). By days 18-20, the yolk sac rapidly shrinks (Marrable, 1971) and, microvilli disappear from the surface of the trophoblast cells (Geisert et al., 1982b). The structure of the placenta is formed by the chorion and vascularized by the allantois and is then defined as an organ of attachment and fetal nourishment (Perry, 1981). Around days 18-20, the single cell layer of the trophoblast develops into the chorion (Geisert et al., 1982a) and, days 18-30 is a time of rapid expansion and development of the allantois (Bazer and First, 1983). The allantois fuses with the chorion around days 19-26 forming the allantochorion and, the amnion and chorion join to form the amniochorion (Marrable, 1971).

Uterine Products (Days 1-21)

Steroids

Porcine endometrium from days 9-10 of the estrous cycle contains enzymes responsible for catalyzing the reductive metabolism of P_4 to pregnanolones (Bazer et al., 1982) and pregnanzediols (Hendricks and Tindall, 1971). On days 14-18, endometrium from pregnant gilts is capable of converting P_4 and neutral steroid precursors to small amounts of testosterone, estrone (E_1), estradiol (E_2) and conjugated E_1 and E_2 (Perry et al., 1976; Gadsby and Heap, 1978; Dueben et al., 1979). Steroids produced by the uterine endometrium may be further metabolized by the conceptus.

In a study by Perry and co-workers (1976), the uterus showed little conversion of sulphated estrogens to unconjugated estrogens

and, there was marked sulphation of E_1 and E_2. Enzyme activity was greater in endometrium than in myometrium on a wet weight basis (Perry et al., 1976). Robertson and King (1974) found that estrogens in maternal peripheral plasma and urine during early pregnancy predominate in the conjugated form of E_1, estrone sulfate (E_1S). These researchers found that maternal plasma levels of E_1S rise on days 9-12, are low on day 14 and markedly increase from day 16 of gestation to peak on days 23-30. The formation of E_1S is probably a result of sulphation of unconjugated estrogens in the emdometrium (Perry et al., 1976). Estrogen sulphotransferase is responsible for this process and is related to the release of P_4 by the CL (Pack and Brooks, 1974; Perry et al., 1973; 1976; Gadsby and Heap, 1978). Concentrations of endometrial cytoplasmic estrogen receptors are similar in nonpregnant and pregnant gilts with maximum levels reached during the early or midluteal phase of the cycle (Deaver and Guthrie, 1980).

<u>Prostaglandins</u>

The luteolytic effect of exogenous prostaglandin F_{2a} (PGF_{2a}) has been demonstrated in hysterectomized gilts (Moeljono et al., 1976; Torday et al., 1980); in pseudopregnant gilts (Kraeling et al., 1975); and in pregnant gilts (Diehl and Day, 1974). PGF_{2a} and its analogues are luteolytic in swine only when administered after day 12 post coitus (Hallford et al., 1975; Moeljono et al., 1976; Guthrie and Polge, 1978). Henderson and McNatty (1975) suggested that the porcine CL may remain refractory to PGF_{2a} until LH begins to dissociate from luteal cell membrane receptors. At this time (day 12), conformational changes within luteal cell membranes may facilitate PGF_{2a} binding. The

PGF_{2a} in turn is then proposed to alter the adenyl cyclase system to inhibit P_4 secretion and activate lysosomal enzymes to cause morphological regression of luteal cells (Flint et al., 1982).

Porcine endometrial tissue produces PGF in vitro (Patek and Watson, 1976; Guthrie and Rexroad, 1980). Uterine-ovarian vein plasma PGF concentrations were significantly greater during the period of luteolysis (days 12-17) in nonpregnant gilts (Gleeson and Thorburn, 1973; Killian et al., 1976; Moeljono et al., 1977; Frank et al., 1977) with little accumulation in the uterine lumen (Zavy et al., 1980). Moeljono (1977) showed no change in PGF levels in the uterine-ovarian vein as measured on days 12-25 of pregnancy. He found a lower basal level of PGF in the uterine-ovarian vein of pregnant gilts than for nonpregnant. Total recoverable PGF is greater in uterine flushings from pregnant than nonpregnant gilts and increases markedly on days 10-18 of gestation (Zavy et al., 1980). By monitoring the PGF metabolite 13,14-dihydro-15-keto-PGF (PGFM), it was further shown that the PGF release through the uterine-ovarian vein during the period of luteal regression in nonpregnant gilts was greater than for pregnant gilts and was not the result of a dilution effect of the increasing uterine and ovarian blood flow seen during pregnancy (Shille et al., 1979; Guthrie and Rexroad, 1981; Ford et al., 1982b). Removal of PGF_{2a} by uterine flushings increased estrous cycle length, therefore it was proposed that PGF_{2a} secretion in the uterine lumen was transferred to uterine venous circulation in nonpregnant gilts (LaMotte, 1977). Geisert and others (1982a) demonstrated that blastocyst estrogen increased PGF and PGE within the uterus. Exogenous

estrogen administered during the estrous cycle decreased the uterine release of PGF_{2a} into uterine-ovarian venous blood and increased PGF_{2a} levels in the uterine lumen (Frank et al., 1977; 1978).

Exogenous estrogen was used to mimic blastocyst estrogen synthesis (days 11-12) during early pregnancy but without other contributions from blastocysts (Geisert et al., 1982c). This study provides evidence that estrogen of blastocyst or exogenous origin initiates events leading to increases in protein, calcium (Ca), PGF and PGE within the uterine lumen. Day 16 endometrial slices have been shown to metabolize arachidonic acid in vitro (Lewis and Waterman, 1982). Blastocyst estrogen may stimulate uterine epithelial secretion through Ca activation of phospholipase A_2 (PLA_2) and, in turn the arachidonic acid cascade may enhance PG synthesis and induce formation of secretory vesicles in endometrial glandular epithelium (Geisert et al., 1982c).

Bazer and Thatcher (1977) proposed a theory of maternal recognition of pregnancy in swine. In the nonpregnant gilt, P_4 enhances PGF_{2a} synthesis by the uterine endometrium and secretion is associated with increased follicular E_2 produced on days 12-18 of the estrous cycle. Secretion of PGF_{2a} during the late luteal phase of the estrous cycle is toward the endometrial stroma and uterine venous drainage (endocrine direction). PGF_{2a} reaching the Cl leads to luteolysis and a return to estrus. In pregnant gilts, P_4 also enhances PGF_{2a} synthesis by the uterine endometrium. That secretion is markedly enhanced by estrogens produced by blastocysts. However, estrogens produced by the blastocyst also affect the direction of secretion of

PGF_{2a} so that it accumulates within the uterine lumen (exocrine direction). Therefore, PGF_{2a} is prevented from gaining access to the CL to exert a luteolytic effect. The direction of movement of PGF_{2a}, like histotroph (Zavy et al., 1980), is determined by the local concentration of estrogen established by the conceptus in the pregnant gilt.

Proteins

Uterine secretions of the estrous cycle and pregnancy have been shown to be regulated by P_4 acting synergistically with estrogen (Knight et al., 1973b; 1974; Roberts et al., 1976; Adams et al., 1981; Fazleabas et al., 1982; Hansen et al., 1985). Characteristic endometrial secretory proteins appear in uterine flushings during the luteal phase of the estrous cycle when P_4 levels are high (Murray et al., 1972; Squire et al., 1972; Knight et al., 1973a). They are also produced in early pregnancy (Zavy et al., 1984) and can be induced in ovariectomized gilts with P_4 treatment (Knight et al., 1973 a,b; Roberts et al., 1976). Uterine secretions contain transport proteins (uteroferrin, retinol and retinoic acid binding proteins), hydrolytic enzymes (lysozyme, cathepsin activities B, D and E, leucine aminopeptidase and other proteases) and protease inhibitors (Roberts et al., 1976; Moffat et al., 1980; Adams et al., 1981; Hansen et al., 1985; Zavy et al., 1984). Enzymatic activity facilitates attachment and the breakdown of macromolecules to allow easier uptake by the conceptus (Hansen et al., 1985). Protease inhibitors which inhibit acrosin, trypsin, chymotrypsin and plasminogen activator (PA) and are secreted by porcine uterine endometrium (Mullins et al., 1980; Basha

et al., 1980; van de Wiel et al., 1985), appear to be taken up by the trophectoderm (Fazleabas et al., 1982) and are believed to protect the uterine epithelium and prevent degradation of nutrients by proteases released by the trophoblast.

Uteroferrin is a purple intrauterine glycoprotein of porcine histotroph (Chen et al., 1973; Basha et al., 1980) that is basic (pI 9.7; Bazer et al., 1975), P_4-induced (Schlosnagle et al., 1974), has a molecular weight of 32000 +/- 3000 and has acid phosphatase activity (Cook and Hunter, 1978). The measurement of acid phosphatase (AP) activity reflects uteroferrin content since 95% of this enzyme activity in the uterus is contributed by uteroferrin (Basha et al., 1979).

In nonpregnant gilts, uteroferrin is secreted into the lumen of uterine glands on days 9-13 of the estrous cycle (Bazer et al., 1982). However by day 15, it begins to become localized within the uterine endometrium stroma surrounding the basement membrane of the uterine glands (Chen et al., 1975 a,b). It has not been found in endometrial stroma in pregnant gilts (Bazer et al., 1982). In pregnancy, uteroferrin is localized within: (1) surface and glandular epithelial cells where it is synthesized and secreted, (2) the lumen of uterine glands and, (3) placental areolae after day 30 of pregnancy (Chen et al., 1975 a,b; Renegar et al., 1982). It is available to transport maternal iron to the developing porcine conceptus for the major portion of gestation (Bazer et al., 1982).

Conceptus Products (Days 1-21)

Steroids

Estrogens are involved in day 7 intrauterine migration and spacing of blastocysts (Pope et al., 1982a). Exogenous estrogens administered on days 11-15 to nonpregnant gilts are luteotrophic (Gardner et al., 1963; Ford et al., 1982a). Day 16-25 embryo extracts exert a luteotrophic effect (Longnecker and Day, 1972; Ball and Day, 1982a,b). In the swine conceptus, there is evidence for an active component that is a steroid rather than a protein because it can be adsorbed by charcoal and is not denatured by heat (Ball and Day, 1982a,b).

Perry and others (1973) first demonstrated E_1 and E_2 production in pre- and peri-attachment porcine blastocysts from neutral steroid precursors, P_4 and E_1S. Later blastocysts were also shown to secrete estriol (E_3; Dueben et al., 1979). Perry and others (1976) showed that day 12 blastocysts produced E_1 in amounts ten-fold greater than estradiol-17B. Estrogen synthesis by the blastocyst is initiated about day 10 and is early enough to affect the signal to the CL (Perry et al., 1973; 1976; Heap et al., 1979; Gadsby et al., 1980). Also, estrogens are found in the trophectoderm and yolk sac endoderm on days 10-16 (King and Ackerley, 1985). Estrogen enzymatic activity is found as early as day 10 (Heap et al., 1975) and all enzymes of estrogen synthesis from pregnenolone are measurable in the blastocyst starting on day 12 and declining thereafter (Perry and Heap, 1973; Flood, 1974; Gadsby et al., 1976; Heap et al., 1981). Pope and others (1982b) showed a 10-fold increase in E_2 on day 12 versus day 9 of pregnancy.

Estrogens are found in greater amounts in pregnant compared to nonpregnant uterine flushings (Zavy et al., 1980). E_2 is present in greater amounts than E_1 and in lesser amounts than E_3 in uterine flushings with filamentous blastocysts (Zavy et al., 1980).

Blastocyst estrogens are assumed to be the signal for maternal recognition of pregnancy (Bazer and Thatcher, 1977; Gadsby et al., 1980) and initiation of estrogen production is associated with blastocyst elongation (Heap et al., 1979; Geisert et al., 1982a,b). Geisert and others (1982b) showed that estrogens increase the uterine luminal content of Ca, proteins and PG's and results in a synchronous release of secretory vesicles from glandular epithelium. Morgan and others (1986) induced this response prematurely (day 9) with exogenous estrogens. Administration of estradiol-17B to cyclic gilts decreases uterine-ovarian venous PGF_{2a} levels and increases PGF_{2a} in the uterine lumen reflecting redirection of PGF_{2a} secretion to block its luteolytic effect (Frank et al., 1977; 1978).

During early gestation, estrogens in maternal plasma and urine predominate in the form of E_1S (Robertson and King, 1974). Conversion of blastocyst estrogens by conjugation to a biologically inactive form before entering maternal circulation would suggest a local effect (Geisert et al., 1982a). The free estrogens may act on the uterus to increase uterine blood flow (Ford and Christenson, 1979), water electrolyte movement (Goldstein et al., 1980), maternal recognition phenomenon (Bazer and Thatcher, 1977) and uterine secretory activity (Geisert et al., 1982c). The trophoblast synthesizes estrogens and has very high sulphatase activity allowing hydrolysis of E_1S to E_1 and

E_2 but lacks the sulphotransferase activity to conjugate them (Perry et al., 1976). After exerting a local effect, unconjugated estrogens are conjugated by sulphotransferases in the endometrium and enter the maternal circulation in a biologically inactive form (van de Wiel and Everts, 1977; Heap et al., 1979). Probable target tissues such as the CL have active sulphatases which could regenerate free steroid (Perry et al., 1976; Cook and Hunter, 1978). Perry and others (1976) provided preliminary evidence for the presence of estrogen receptors in porcine CL and showed that luteal cytosol has estrogen binding capacity.

Prostaglandins

Prostaglandins, PGF_{2a} and PGE_2, are produced by porcine blastocysts and probably make a substantial contribution to the uterine luminal milieu of pregnancy (Watson and Patek, 1979; Zavy et al., 1980; Geisert et al., 1982a). Nkuuhe and Manns (1981) demonstrated that PGE_1 influences the flux of fluid into the uterine lumen and the movement of PGF_{2a} across the uterine epithelium in the ewe. The conceptus may also be affected by PG's since they promote cellular proliferation as mediated by cAMP (MacManus and Whitfield, 1974) and stimulate steroid biosynthesis (Batta, 1975).

The conceptus and endometrium metabolize arachidonic acid in vitro but on a wet weight basis, the conceptus metabolizes much more (Lewis and Waterman, 1982). Maule Walker and others (1977) have suggested that the conceptus (amnion in particular) may be involved in metabolizing PGF_2 and PGE_2 to their respective PGFM. It should be noted that total recoverable PGF was only 10226 ng for both horns in pseudopregnant gilts as compared to 27688 ng for both uterine horns

of pregnant gilts (Frank et al., 1978). This may reflect the synthesis of PGF by porcine blastocysts or an increase in uterine activity due to the presence of blastocysts.

PGF and PGE_2 content in uterine flushings increase at the time of blastocyst migration, elongation and estrogen production (Pope et al., 1982b; Geisert et al., 1982a,c). Total PGE_2 is almost 12 times higher in day 12 pregnant flushings containing filamentous blastocysts as compared to day 12 nonpregnant uterine flushings and, this difference is almost 60 times greater for pregnant animals by day 14 (Geisert et al., 1982a).

PG's are synthesized from arachidonic acid with phospholipase A_2 (PLA_2) as the rate limiting enzyme (Davis et al., 1983). Both PLA_2 activity and PG content increase in the 7-14 day blastocyst. Increases in PLA_2 activity and PG content could be accounted for by the rise in the mass of the blastocyst with age. Because estrogen has been shown to stimulate PLA_2 activity in the rat uterus (Dey et al., 1982), it is possible that blastocyst estrogen stimulates PG production either in the blastocyst or in the surrounding endometrium or both through an increase of PLA_2 activity in these tissues (Perry et al., 1973). Geisert and others (1982a,c) showed that estrogen increased PG's in porcine uterine flushings.

<u>Proteins</u>

Wyatt (1976) showed that day 16 trophectoderm incorporation of leucine into proteins was significantly higher when embryonic tissue was cultured together with an endometrium explant than when cultured either alone, or with peritoneum, kidney, liver or striated muscle

explants. This provides evidence that endometrial factors affect protein synthesis in the peri-attachment conceptus.

During the initial stages of blastocyst elongation (days 10.5-12), a group of low molecular weight acidic proteins (Mr 20-25000; pI 5.6-6.2) are most prominent (Godkin et al., 1982a). From days 13-18 a group of basic proteins with molecular weights of approximately 35-50000 are synthesized and released (Godkin et al., 1982a). Powell-Jones and coworkers (1984) also found proteins of lower (14000 and 19000) and higher molecular weights (88000) produced between days 13-16 and attributed this difference from the results of Godkin and others (1982a) to a difference in procedure. The major conceptus proteinaceous product has been identified as having a molecular weight around 45000 and pI 8.0 (Godkin et al., 1982a; Powell-Jones et al., 1984). A high molecular weight glycoprotein (50% carbohydrate; Mr > 600000) is also produced by porcine trophoblast on days 13-16 (Godkin et al., 1982a; Masters et al., 1982; Powell-Jones et al., 1984). The ovine and bovine conceptuses produce a similar glycoprotein which may provide an immunoprotective barrier between the conceptus and maternal lymphocytes or possibly influence the permeability of the endometrium during pregnancy (Godkin et al., 1982b; Masters et al., 1982). This nonsulphated glycoprotein lacks sialic acid but is high in galactose and N-acetylglucosamine and, there are N-linkages between carbohydrate and peptide (Godkin et al., 1981; Masters et al., 1982). These proteins are still present in gilts on day 18 but diminish as new proteins of higher molecular weight (Mr 50-70000) and of varied pI's are produced. By day 20 embryonic polypeptides are more evident than

proteins synthesized by the trophoblast. The day 18, 20 and 25 embryo and yolk sac synthesize and release major polypeptides in the 50-70000 molecular weight range that correspond in pI's and electrophoretic mobilities with the six major fetal plasma proteins (Buhi et al., 1982) including transferrin, a fetoprotein, and fetuin (Godkin et al., 1982a).

Between days 10-16, porcine blastocysts biphasically release the serine protease, plasminogen activator (PA), which appears to be regulated by estrogen (Mullins et al., 1980; Zavy et al., 1982) and is also present in the pseudopregnant uterus (Mullins et al., 1980; Fazleabas et al., 1982). Although PA is involved in the invasive growth of cells, porcine blastocysts do not undergo invasive implantation in the uterus. The porcine uterus secretes a P_4 induced plasmin inhibitor (Mullins et al., 1980; Fazleabas et al., 1982). The initial phase of PA release (days 10-12) coincides with early elongation of the blastocyst and may act within the conceptus to promote cellular remodelling. The second phase (days 14-16) occurs during a time at which DNA content of the blastocyst is increasing markedly and tissue is proliferating for yolk sac development (Fazleabas et al., 1983). The zymogen substrate for PA, plasminogen, is present in highest amounts on day 12 when there is a depression in PA production by the blastocyst and the production of plasmin inhibitor is at its peak (Fazleabas et al., 1983; Chaichimansour, et al., 1986).

Acrosin inhibitor production by the uterus was discussed earlier. However, day 18 blastocyst production of acrosin inhibitor was found

to be 4 to 5 times higher than from uterine mucosa tissue taken at the corresponding attachment site (van de Wiel et al., 1985).

CHAPTER 3

MATERIALS AND METHODS

Animals

Crossbred gilts (Duroc, Hampshire, Landrace and Yorkshire breeding) were maintained in outside pasture lots located at the Blount Experiment Station in Knoxville, Tennessee and checked daily for estrus in the presence of an intact boar. Gilts were bred naturally or artificially upon detection of standing estrus and again 24 hours later. The first day of standing estrus was designated as day 0. Gilts were fed approximately 2.2 kg of a 16% crude protein corn-soybean meal diet once a day and, water was provided *ad libitum*.

Conceptus Recovery and Incubation

Surgical collection of conceptus material was performed on day 16+/-1 post-breeding. The gilts were initially anesthetized by ear vein injection of sodium thiamylal (1 g/20 ml 0.9% NaCl), and anesthesia was maintained during surgery by closed circuit halathane (Fluothane, Fort Dodge Laboratories, Fort Dodge, IA) administration by a gas inhalation machine. Under aseptic conditions, the reproductive tract was exposed via midventral incision, ligated and removed. The uteri were transferred to a sterile laminar flow hood, and infused with approximately 60 ml Hanks' balanced salt solution containing 2% (v/v) antibiotic-antimycotic (ABAM) injected via an 18 guage needle inserted near the uterine body. The uterine horns were massaged and the flushing medium retrieved into sterile culture dishes from an

incision made at the tubouterine junction. Each uterine horn was flushed separately.

Conceptus material was transferred to sterile Petri dishes containing 15 ml Eagle's minimum essential medium (MEM) supplemented with 2% (v/v) ABAM, 1% (v/v) MEM vitamin solution, 1% (v/v) MEM non-essential amino acids, 0.2 units insulin and 100 uCi L-[4,5 ^{3}H] leucine (ICN Radiochemicals, Irvine, CA). The radiolabelled leucine was used as a tracer of conceptus synthesized proteins released into the culture medium. The dishes were transferred to a controlled atmosphere chamber which was then flushed for 1-2 minutes with a gaseous mixture containing 50% O_2, 45% N_2 and 5% CO_2 by volume. There the conceptuses were incubated in the dark for 24 hours on a rocker at 37 C. This method was originally developed by Barrett and coworkers (1976) for the incubation of bronchial tissue explants and more recently adapted for the incubation of porcine endometrium (Basha et al., 1979) and porcine conceptuses (Godkin et al., 1982a). Conceptuses and culture medium were then centrifuged at 12100 x g for 15 minutes at 4 C to separate tissue and medium. The supernatant was removed and frozen at -20 C and the pellet was discarded. All tissue culture reagents used above were purchased from Gibco Life Technologies, Inc., Chagrin Falls, OH unless stated otherwise.

<u>Protein Purification and Characterization</u>

Culture medium was dialyzed (4 C) against 4 liters of 10mM sodium acetate buffer solution at pH 5.4 using dialysis tubing with a 6000-8000 molecular weight cut off to remove low molecular weight compounds. The buffer solution was replaced three times; each time

after approximately 8 hours of dialysis. Dialyzed media were then combined and applied to a 2 x 8 cm carboxymethylcellulose (CMC; Pierce Chemical Co., Rockford, IL) column equilibrated with 10mM sodium acetate buffer, pH 5.4, to bind basic proteins. The bound basic proteins were eluted with a 300 ml linear salt gradient (0-0.5 M NaCl) and collected in 80 four ml fractions. Two 20 ul aliquots of each fraction were pipetted into counting vials and 4 ml of Scinti Verse II scintillation cocktail (Fisher Scientific Co., Fair Lawn, NJ) were added to each vial. The radioactivity (^3H) was measured in a ISOCAP 300 Searle liquid scintillation counter (model 6868). The collected fractions were then pooled according to radioactive peaks. Peak III material was then dialyzed as before to remove salt applied to the CMC column and, the recovered dialyzate was then concentrated over a 1 x 1 cm CMC column. After the column material and bound proteins were transferred to a centrifuge tube, four ml of 1M NaCl in 10mM sodium acetate buffer was vortexed into the CMC slurry. The mixture was then centrifuged at 1000 x g for 5 minutes at 4 C to elute the bound proteins into the supernatant. The concentrated fractions were combined (4-12 ml) and applied to a 2.5 x 90 cm Sephacryl-200 (S-200; Pharmacia Fine Chemicals, Piscataway, NJ) column equilibrated with 10 mM Tris HCl, 0.3 M NaCl and 0.02% NaN_3 at pH 8.2. The fractions forming the single peak were pooled, dialyzed, and concentrated over another 1 x 1 cm CMC column.

Following dialysis against 500 ml of 10 mM sodium acetate at pH 7.0, protein concentration was estimated by the method of Lowry and coworkers (1951). Bovine serum albumin (BSA) was used as the

standard. One ml of each dialyzed concentrated sample was lyophilized in preparation for characterization by non-equilibrium pH gradient electrophoresis (NEPHGE) developed by O'Farrell and others (1977). NEPHGE is a modification of the two dimensional polyacrylamide gel electrophoresis (2D PAGE) procedure (O'Farrell, 1975) as described by Horst and Roberts (1979) and Horst and coworkers (1980) that can resolve basic as well as acidic polypeptides. Protein samples were solubilized according to Horst and others (1980). Electrophoresis protocol is outlined in the Appendix. Protein standards (Sigma Chemical Co., St. Louis, MO) used were transferrin (Mr 78000), ovalbumin (Mr 43000), chymotrypsinogen (Mr 25000) and ribonuclease A (Mr 13899). After electrophoresis, the acrylamide slabs were fixed in 53% water, 40% ethanol and 7% acetic acid, stained with Coomassie Brilliant Blue R and destained in 83% water, 10% acetic acid and 7% ethanol. After soaking the slabs 30 minutes in 1M sodium salicylate (Chamberlain, 1979) and drying, radioactivity was detected by fluorographs prepared from exposure to Kodak XAR-5 X-ray film at -70 C for 2-4 weeks.

Rabbit Immunization and Antibody Detection

Three ml of the concentrated protein sample (720 ug) was lyophilized, solubilized in 1 ml deionized (DI) water, emulsified in 1 ml Freund's complete adjuvant (Sigma Chemical Co., St. Louis, MO) and then injected subcutaneously in multiple sites along the back of a 7 month old female rabbit. A booster injection containing lyophilized antigen (160 ug) brought up to 1 ml in DI water and emulsified with 1 ml Freund's incomplete adjuvant (Sigma Chemical Co., St. Louis, MO)

was injected four weeks later. A 5 ml blood sample was taken from the ear vein of the rabbit prior to each injection.

To remove clotting factors, blood samples were allowed to sit overnight at 4 C and then were centrifuged three times at 1000 x g for 15 minutes at 4 C. Serum was tested for the presence of antibody (Ab) by Ouchterlony agar gel immunodiffusion (Garvey et al., 1977). Tissue culture dishes (35 x 10 mm) were half-filled with 3 ml of 1% (w/v) agarose made up in phosphate buffered saline solution (PBS). Basic conceptus protein (400 ug/ml) was used at full, half and quarter strength in 18 ul wells, however no precipitin bands were indicated. Two weeks later, a 50 ml blood sample was procured from the treated rabbit. Ouchterlony plates using 510 ug Ag/ml, in the same serial dilutions as before, showed the presence of new antigen-antibody (Ag-Ab) complexes by the formation of precipitin lines.

Immunoprecipitation

In preparation for Ag-Ab complex collection with Protein A-Sepharose, aliquots of antiserum, pre-immune serum (collected prior to immunization) and cultured medium containing ^{35}S labelled total conceptus protein were centrifuged at 4 C for 15 minutes at 12000 x g and, the pellets were discarded. Fifteen ul of antiserum or pre-immune serum were added to 1 ml of medium and incubated at 4 C overnight on a rocking platform. After centrifugation at 4 C for 15 minutes (12000 x g), the pellets were discarded and 200 ul of a 10% suspension of Protein A-Sepharose were added to the supernatants. After incubating 4 hours at 37 C on a rocker, the beads were washed 6 times with 1 ml of PBS pH 7.4 containing 0.02% sodium azide and 0.1%

BSA to remove nonspecific Ab and once with DI water to remove salt which would precipitate with sodium dodecylsulfate (SDS). The pellets were solubilized in 40 ul of 5% SDS, 5 mM Tris, pH 6.8, 15% glycerol and 2 ul of B-mercaptoethanol (B-ME), boiled 3 minutes and centrifuged for 15 minutes (12000 x g). The supernatants (40 ul) were loaded onto a gel and submitted to 1D PAGE according to the method of Laemmli (1970). Electrophoresis protocol is outlined in the Appendix. Slabs were prepared and radioactivity was detected by fluorographs as described earlier.

Crossreactivity

Endometrial, amnionic and chorionic tissues were surgically collected from gilts on day 111 of pregnancy. The tissues were incubated as described earlier for conceptus tissues however, ^{35}S methionine was used as the tracer. Tissue incubation medium and fluids collected during surgery were centrifuged at 12100 x g for 15 minutes at 4 C. The supernatant was removed and the pellet was discarded. Collected fluids and incubation medium were dialyzed at 4 C against PBS at pH 7.4 as previously described. Ten ml of each fluid sample was then lyophilized and brought up in 1 ml of PBS.

The antiserum was tested for crossreactivity with the above labelled tissue incubation medium, amnionic (day 90) and allantoic fluids (days 90 and 111), total porcine conceptus protein (days 15-17), uterine flushings from pseudopregnant gilts and peripheral plasma collected from a nonpregnant gilt by Ouchterlony immunodiffusion as described earlier. The previously described immunoprecipitation procedure was employed to more accurately determine crossreactivity of

the antiserum to the ^{35}S labelled proteins found in endometrial, amnionic and chorionic tissue culture medium.

CHAPTER 4

RESULTS AND DISCUSSION

Isolation of Protein

When dialyzed conceptus incubation medium were submitted to CMC ion exchange chromatography, basic proteins were bound by the gel while acidic proteins passed through. The bound proteins were then eluted with a linear NaCl gradient into three prominent peaks (Figure 1) similar to results of a previous study by Godkin and others (1982a). This earlier work showed peak III to contain the major single protein produced by the day 14-16 porcine conceptus. Therefore, the CMC peak III material obtained in the present study was submitted to S-200 gel filtration chromatography for separation by molecular weight and, a single peak of radioactivity was distinguished (Figure 2). Protein content of concentrated samples was estimated by the method of Lowry and coworkers (1951) to ensure that at least 50 ug of protein were loaded onto each electrophoresis gel. Fluorography of dried NEPHGE gels of the S-200 peak material revealed only one protein (Mr 43000, pI 7.5-8.0) indicating that it was radiochemically pure (Figure 3).

Development of Antiserum

Antiserum (18 ul), collected from rabbits immunized with S-200 peak protein, precipitated against 9.180 ug, 4.590 ug and 2.295 ug of S-200 peak protein as demonstrated by Ouchterlony immunodiffusion analysis. For a more accurate characterization of antiserum, total conceptus protein and antiserum were incubated overnight and Ag-Ab

CRANE'S CREST

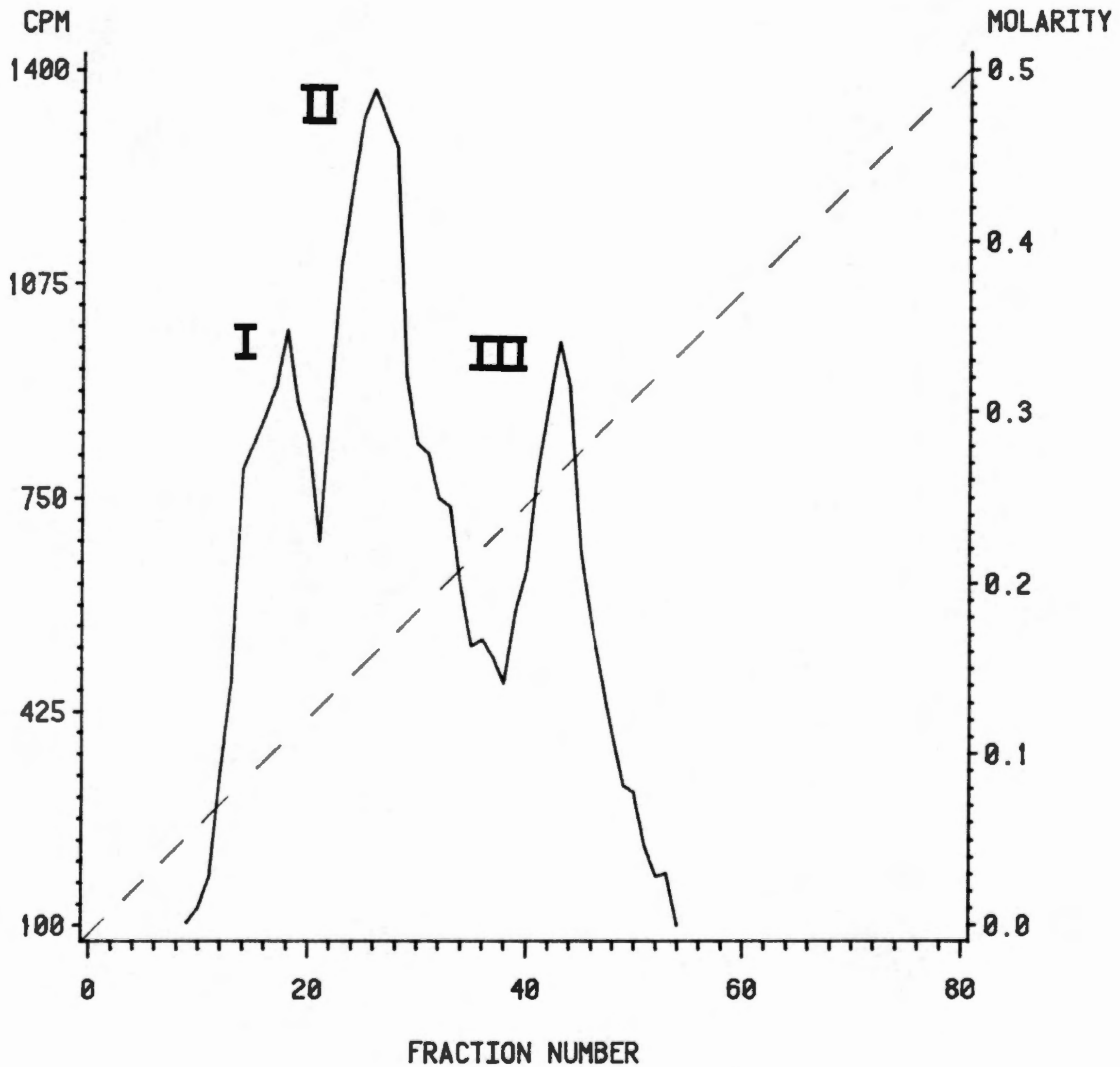


Figure 1. Carboxymethylcellulose ion exchange chromatography of dialyzed conceptus culture medium. Following separation from acidic proteins, basic proteins were eluted with a linear NaCl gradient into three prominent peaks. The broken line shows the concentration of NaCl.

CRANE'S CREST

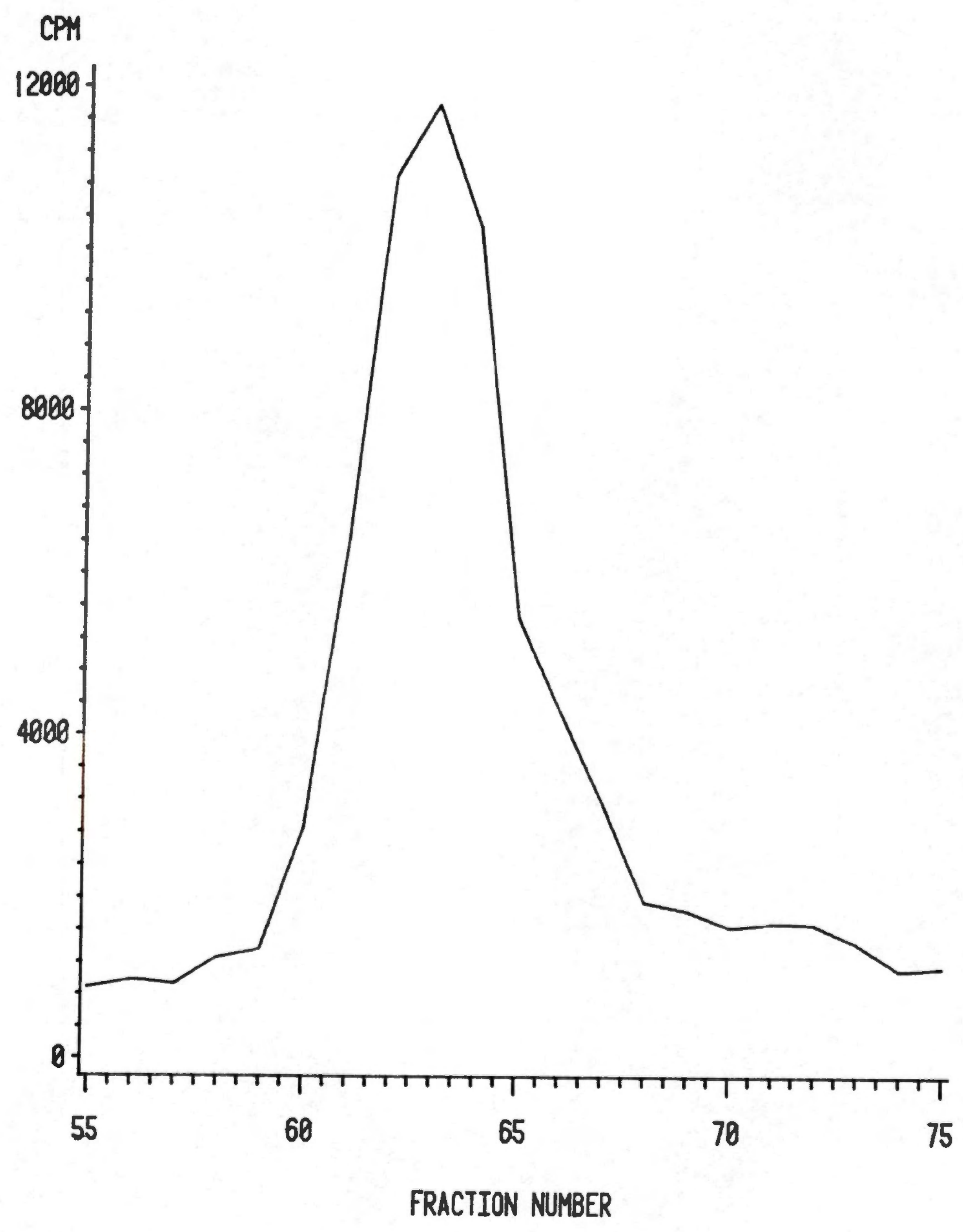


Figure 2. Sephacryl-200 gel filtration chromatography of peak III from carboxymethylcellulose ion exchange chromatography. CMC peak III material was dialyzed and concentrated before being loaded onto a S-200 column.

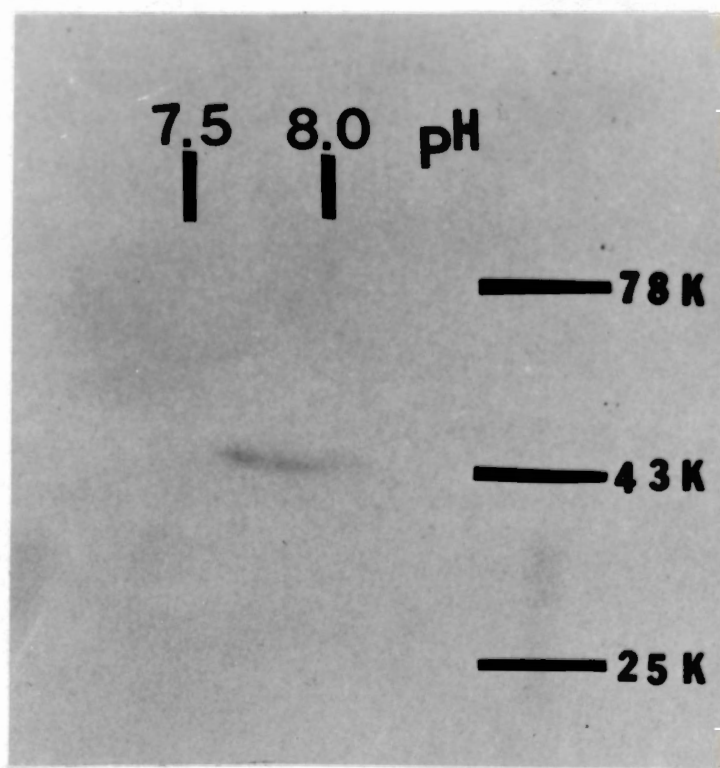


Figure 3. Fluorography of Sephacryl-200 peak fractions following non-equilibrium pH gradient electrophoresis. The S-200 peak of radioactivity was submitted to NEPHGE revealing only one protein of approximate molecular weight of 43000 and pI 7.5-8.0.

complexes were collected with Protein A-Sepharose. Ag-Ab complexes were loaded onto a 1D PAGE gel (Figure 4a) and submitted to fluorography (Figure 4b).

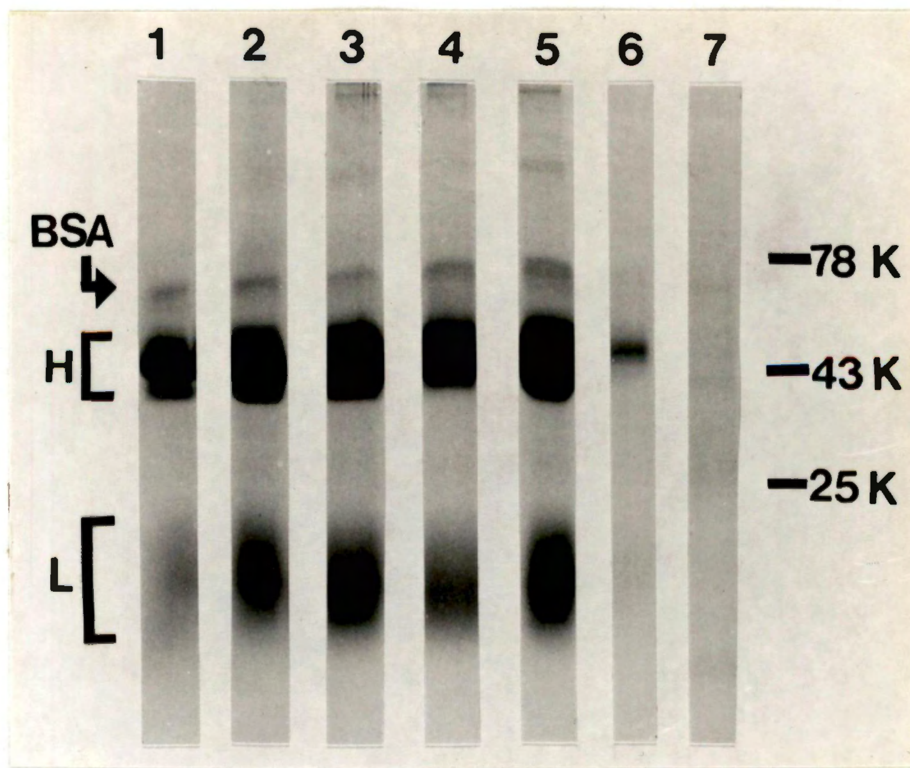
Figure 4a illustrates the Ag-Ab complexes formed from incubation of 5 ul, 10 ul, 20 ul and 40 ul (lanes 1-4, respectively) of antiserum each with 300 ul total conceptus protein. Duplicate samples of the Ag-Ab complexes with different dilutions of antiserum measured 1090, 1376, 2299 and 4341 cpm, respectively. Ag-Ab complexes (206 cpm) formed from incubation of pre-immune serum and total conceptus protein were loaded into lane 5. Lanes 6 and 7 contained S-200 peak protein and total conceptus protein, respectively. As seen in Figure 4a, lanes 1-5 of the gel exhibited the Coomassie blue stained heavy (H) and light (L) chains of rabbit immunoglobulin G (IgG), as well as BSA, which was used to remove nonspecific Ab. The single band in lane 6 confirmed that the purified S-200 peak protein was approximately 43000 in molecular weight. The total conceptus protein did not stain well in lane 7 of the gel.

Fluorography of the gel was performed in order to visualize the immunoprecipitates formed with radioactive protein (Figure 4b). It is unknown why lane 4 showed no crossreactivity of antiserum with other conceptus proteins while incubations with lower amounts of antiserum (lanes 1, 2 and 3) revealed two other higher molecular weight bands. The major Ag-Ab complex in lanes 1, 2 and 3 and the only band in lane 4 is at the same molecular weight as the conceptus protein in major production (lane 7) and the S-200 peak material stained in lane 6 of the gel (Figure 4a). The ^{3}H labelled purified protein in lane 6 did

Figure 4. Electrophoretic analysis and fluorography of total conceptus protein immunoprecipitates.

4a. Electrophoretic analysis of total conceptus protein immunoprecipitates. The immunoprecipitate reaction using rabbit anti-S-200 peak protein serum and total conceptus protein was solubilized and separated by 1D PAGE in a 10% acrylamide gel. Gels were fixed and stained in Coomassie Brilliant Blue R. Ag-Ab complexes formed from incubation of 5 ul, 10 ul, 20 ul and 40 ul of antiserum each with 300 ul total conceptus protein were loaded into lanes 1, 2, 3 and 4. Pre-immune serum incubated with total conceptus protein was loaded into lane 5. The S-200 peak protein is presented in lane 6. Total conceptus protein (lane 7) did not stain well. The heavy (H) and light (L) chains of rabbit immunoglobulin G(IgG) and BSA are indicated.

4b. Fluorography of one dimensional polyacrylamide gel electrophoresis of total conceptus protein immunoprecipitates. The major radioactive Ag-Ab complexes measuring approximately 1090 cpm (lane 1), 1376 cpm (lane 2), 2299 cpm (lane 3) and 4341 cpm (lane 4) are the same molecular weight as the major conceptus protein present in total conceptus protein (lane 7). The 2 higher molecular weight bands seen in incubations with smaller amounts of antiserum (lanes 1, 2 and 3) were not produced in lane 4. Pre-immune serum did not form a complex with any radioactive conceptus proteins as seen in lane 5. The ^{3}H labelled S-200 peak protein (lane 6) did not form a band on the fluorograph because of a short exposure time to the film.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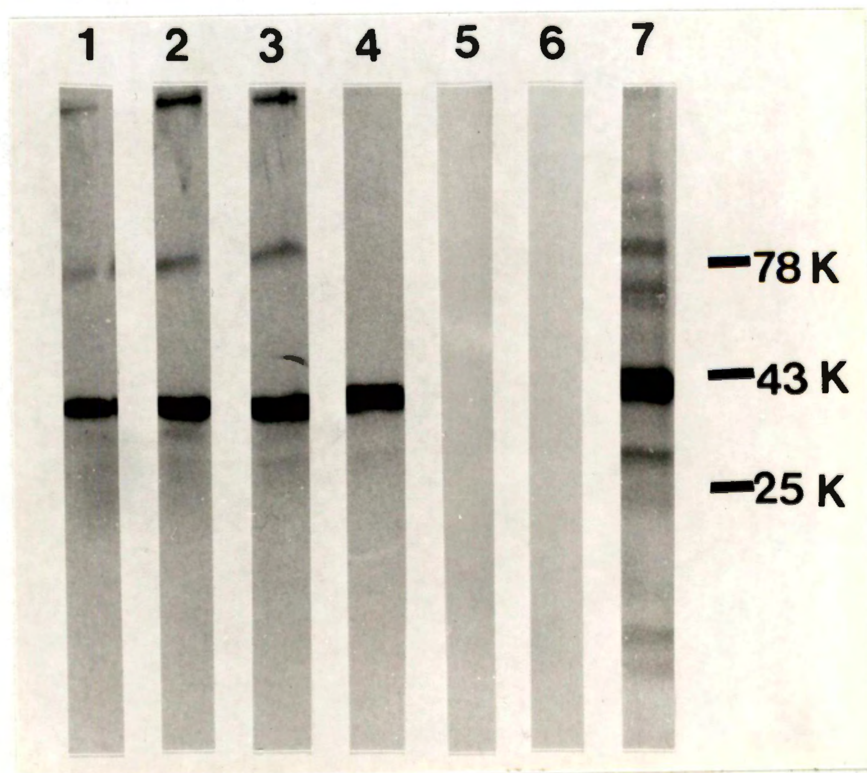
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not show up on the fluorograph because the film was only exposed to the gel long enough to show the ^{35}S methionine present in other lanes. Pre-immune serum did not form Ag-Ab complexes with any radioactive conceptus proteins (lane 5; Figure 4b).

Characterization of Antiserum

Characterization of antiserum by Ouchterlony immunodiffusion showed that the antiserum does not crossreact with: (1) porcine allantoic fluid obtained from gilts on days 90 (230 ug) and 111 (265 ug) of gestation; (2) porcine amnionic fluid (155 ug) from day 90 of gestation; (3) uterine flushings (202 ug) from pseudopregnant gilts; (4) peripheral plasma from a nonpregnant gilt; (5) total porcine conceptus protein (9.5 ug; days 15-17); (6) porcine endometrial (26 ug), amnionic (5 ug) and chorionic tissue (27 ug; day 111 of gestation) incubation medium. Antiserum should have precipitated with the major protein present in total conceptus protein. However, the major protein was probably present in too small an amount to form visible precipitin lines with the antiserum.

Following immunoprecipitation of antiserum with endometrial (lane 1), amnionic (lane 2) and chorionic (lane 3) tissue medium and total conceptus protein (lane 4), the samples were submitted to 1D PAGE. Fluorography (Figure 5) of the gel demonstrated that the antiserum did not crossreact with any protein other than the major conceptus protein (lane 4). Lane 5 contains total conceptus protein. The conceptus protein in major production (lane 5) is the same molecular weight as the Ag-Ab complex in lane 4. Since the major conceptus protein does not seem to be present in the peripheral circulation of nonpregnant

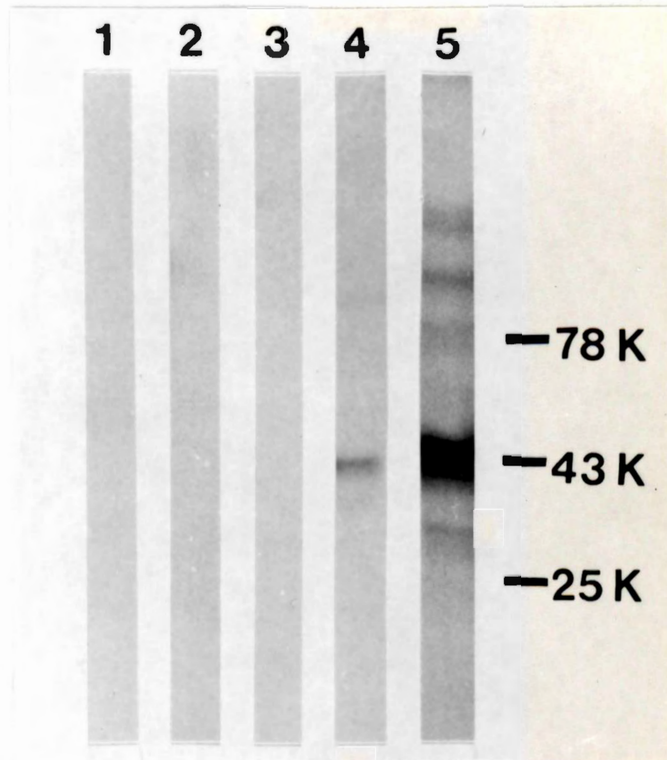

Figure 5. Fluorography of one dimensional polyacrylamide gel electrophoresis of crossreactivity of antiserum with endometrial, amnionic and chorionic culture medium. No crossreactivity was evident with endometrial (720 ug; lane 1), amnionic (125 ug; lane 2) and chorionic (750 ug; lane 3) produced proteins from late pregnancy. Immunoprecipitate only formed with total conceptus protein (265 ug; lane 4). Total conceptus protein in lane 5 show that the protein in major production days 15-17 of gestation and the Ag-Ab complex in lane 4 have approximate molecular weights of 43000.

gilts or produced by late pregnant or E_2 primed nonpregnant maternal tissues or by fetal tissues in late pregnancy, it is likely that it is only produced by the peri-attachment porcine conceptus.

Importance of Conceptus Proteins

During the period of maternal recognition of pregnancy in ewes, cows and gilts, there is a group of low molecular weight acidic proteins released from the conceptus (Godkin et al., 1982a,b; Bartol et al., 1985). These proteins have been determined to be antiluteolytic in both ewes (Martal et al., 1979; Godkin et al., 1984b) and cows (Knickerbocker et al., 1986) and to stimulate uterine secretion of proteins in the ewe (Godkin et al., 1984a; Salamonsen et al., 1986). The major ovine trophoblast protein, oTP1, appears to be similar to some of the conceptus proteins seen in cattle (Helmer et al., 1985). Heat treatment or charcoal adsorption of conceptuses or conceptus extracts indicate that conceptus proteins are the antiluteolytic substance in ewes and cows (Rowson and Moor, 1967; Martal et al., 1979) whereas estrogen may be the major antiluteolytic substance in gilts (Ball and Day, 1982a).

Basic proteins similar to those produced by the peri-attachment porcine conceptus are not found in sheep (Godkin et al., 1982b) or cattle (Bartol et al., 1985). Godkin and others (1982a) were the first to demonstrate that production of the major porcine conceptus protein during the peri-attachment period begins about day 13, peaks on day 16, declines on days 18-20 (coninciding with completion of attachment) and is not present on days 25 or 30 of gestation. In the present study, antiserum did not bind proteins produced by the day 90 or 111

endometrium, amnion or chorion. Through the use of protein purification techniques and antiserum to the major conceptus protein, the function of this protein may now be further characterized without interference from other proteins of conceptus or uterine origin.

Godkin and coworkers (1984a) infused ^{125}I-labelled oTP1 into the uterine lumen of nonpregnant ewes and determined that very little of the protein entered the maternal circulation but was retained within the uterus. In this same study, immunocytochemical techniques were employed and, it was found that oTP1 becomes associated with the luminal surface of uterine epithelial cells and the epithelium of upper uterine glands but is not found in deep glands, stroma or muscle layers of the uterus or ovarian, CL or other extrauterine tissues. This study also showed that oTP1 receptors were found in pregnant and nonpregnant endometrium and that oTP1 does not directly stimulate P_4 production by luteal cells. Now that an antiserum to the major porcine conceptus protein has been developed, similar immunocytochemistry techniques can be employed to study uterine binding of conceptus protein in swine.

If it is determined that the major basic conceptus protein exerts a local effect upon the porcine uterus, then it would be beneficial to try to quantitate the amount of protein present to elicit a uterine response. The amount of protein released into culture media by day 16 conceptuses and the wet weight of the conceptus tissue could be used to estimate the amount of protein present in an entire uterus. However, conceptuses in vitro may secrete far less protein than conceptuses in vivo. Removal of a section of pregnant uterus

containing conceptuses and uterine fluid would allow a more accurate measurement of protein content within a certain length of pregnant uterus. This information may suggest a minimum amount of protein necessary to maintain early embryo survival.

Daily infusion of the major basic porcine conceptus protein into a segment of the pseudopregnant porcine uterus or, antiserum into a pregnant uterus, could show the effect of the presence or absence of the major conceptus protein on the maintenance of pregnancy. CL function or the uterine secretion of proteins, calcium or PG's may be influenced by the presence of this conceptus protein in the uterus. Alternately, a comparison of secretions from uterine tissue explants of nonpregnant, pseudopregnant and pregnant gilts cultured with and without the major basic conceptus protein may show a difference in the character and amount of uterine secretions. In this way, less protein would be required for incubation of conceptus protein with uterine explants than that needed for in vivo studies. Use of day 12 pregnant uterine tissue in the above mentioned studies instead of tissue from pseudopregnant gilts would be possible because maternal recognition of pregnancy would have already occurred but the major basic conceptus protein would not be present. Uterine infusions and incubations of uterine tissue with total basic conceptus proteins may determine whether the major basic conceptus protein is part of a complex or acts alone. Determination of the site of action and function of the major conceptus protein produced during attachment will aide in understanding maternal-conceptus interactions during a period when the embryonic mortality rate is at its highest in swine.

CHAPTER 5

SUMMARY

Proteins released by the day 15-17 porcine conceptus in vitro were subjected to carboxymethylcellulose ion exchange and Sephacryl-200 gel filtration chromatography. Isolation of the major basic conceptus protein (Mr 43000; pI 7.5-8.0) from other components of conceptus secretion was verified by non-equilibrium pH gradient electrophoresis and fluorography.

Antiserum against the major basic porcine conceptus protein was developed in the rabbit. Specificity of the antiserum was determined by Ouchterlony immunodiffusion, antigen-antibody immunoprecipitation, one dimensional polyacrylamide gel electrophoresis and fluorography. The molecular weights of the labelled Ag-Ab complex and the peri-attachment conceptus protein in major production are approximately 43000. The antiserum did not crossreact with 1) uterine flushings from pseudopregnant gilts, 2) peripheral plasma from nonpregnant gilts, or 3) amnionic and allantoic fluid and media from cultures of endometrial, chorionic or amnionic tissue from late pregnant gilts. This antiserum will be used in future studies designed to examine the site of action and function of the major protein product of the peri-attachment porcine conceptus.

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LITERATURE CITED

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APPENDIX

APPENDIX

PROCEDURE FOR ONE-DIMENSIONAL POLYACRYLAMIDE GEL ELECTROPHORESIS (1D PAGE)

A. Preparation of Slabs

Clean glass plates thoroughly, rinsing well. Rinse with alcohol to remove grease. Rinse again with DI water.

To prepare glass plate sandwiches:

Lay plate on flat surface so one edge overhangs. Place spacers on opposite margins. Put another glass plate directly on top of first, making sure that all edges are flush and put the clamps on the overhanging side. Tighten screws. Put gaskets in stand with ridges fitting into grooves. Level casting stand. Place sandwich in stand with screws facing outward and bottom edge of plate against solid gasket. With ridges facing up insert cams into holes on sides of stand and then turn both at once 180 degrees. Seal bottom edge of glass to gasket with agarose.

B. Running Gel Preparation for Slabs

Running Gel A-Acrylamide and Buffer B are removed from refrigerator and allowed to warm to room temperature.

Mix according to following recipe for 10% SDS Slabs:

	2 GELS
Acrylamide Buffer A	28.0 ml
Buffer B	21.0 ml
20% SDS	420.0 ul
water	16.6 ml
APS (made fresh daily)	18.0 ml

Thoroughly mix first four ingredients then add APS and swirl to mix.

Use a pipet or syringe to fill sandwich up to 1 cm below comb, avoiding turbulent flow. Tap stand on lab bench to cause any air to float to surface. Using a glass pipet overlay with 10% SDS to 3 mm in depth evenly over top of slab. Allow to polymerize 30 minutes before adding stacker and 2 hours before running. DO NOT USE A GEL POLYMERIZING IN LESS THAN 10 MINUTES OR GREATER THAN 30 MINUTES.

C. Stacking Gel Preparation

Pour off SDS and rinse with water; repeat twice and blot with paper towel.

Stacking Gel

	2 GELS
Buffer A-Acrylamide	2.4 ml
Buffer E	2.0 ml
20% SDS	80.0 ul
water	6.3 ml
APS	5.3 ml
TEMED	5.0 ul

Thoroughly mix first five ingredients then add APS and TEMED. Swirling to mix.

Put comb between glass plates. Add stacking gel to top of glass plates being careful not to trap air under teeth. Allow to polymerize 15-30 minutes. Remove comb slowly straight up to avoid tearing gel. Rinse wells with electrode buffer to remove small fragments of polyacrylamide and unpolymerized monomer. Fit slotted silicone gaskets into the upper side of the upper buffer chamber and place on top of sandwich in casting stand. Remove cams from casting stand and place ridge down into holes in buffer chamber and rotate 180 degrees. Turn upside down to drain and seal glass to top gasket with agarose. To apply sample, fill each well with electrode buffer. Use needle and syringe to underlay sample in each well.

D. Preparation of Sample for Wells

Dissolve lyopholized sample in least amount of solubilizing buffer:

	for 100 ml:
20mM Tris acetate	2 ml 1M Tris
20% glycerol	20 ml glycerol
20% SDS	4 ml 20% SDS

For each 10 ml of the above, add 200 ul 2% B-ME just before use and a drop of bromophenol blue as tracker.

E. Electrophoresis

Place the lower buffer chamber with a stir bar on a stir plate. Place heat exchanger with hoses attached inside chamber. Fill lower chamber with electrode buffer. Place upper buffer chamber and plates in lower buffer chamber. Add buffer to top chamber slowly until central ridge is covered (600 ml). Put lid on cell, connect leads, plug electrodes into power unit and turn on power unit, coolant and stir plate.

Power unit is on constant current 15 ma/slab for 2 hours to allow for stacking, then 20 ma/slab until dye band reaches around 5 mm from bottom.

F. Removal of Slab Gels

Turn off current, coolant, and stir plate. Disconnect leads from power unit. Lift out unit and empty upper buffer chamber. Remove cams and upper buffer chamber from sandwiches, then loosen screw clamps and remove. Lift corner of plate gently with wooden or teflon spatula to admit air.

PROCEDURE FOR NON-EQUILIBRIUM pH GRADIENT ELECTROPHORESIS (NEPHGE)

A. Focusing Gel Tubes

Heavy walled tubes, 14-15 cm long, inner diameter 2.5 mm with ends slightly fire polished.

Clean thoroughly the day before by boiling in concentrated nitric acid. Rinse, siliconize with 1% solution Aqua Sil (5 ml Aqua Sil + 99 ml water, prepared fresh). Rinse, dry in oven.

B. Focus Mix -- prepare fresh each time

Add 5.5 g high purity urea (9.3 M) to a clean conical tube. This will make enough for 9 gels.

Add 1.50 ml Acrylamide-DATD stock solution and 2 ml NP-40 (10% v/v water). Bring this solution up to about 9 ml with water (for 9 gels). Dissolve by running warm tap water over tube and mixing by shaking. Try to avoid excess foaming.

Add the following:

pH 9-11 Ampholine	0.35 ml
pH 8-9.5 Ampholine	0.10 ml
pH 6-8 Ampholine	0.05 ml

The ampholine should be removed in a sterile manner with a Hamiltion syringe or 1 cc Tuberculine Syringe.

De-gas mixture for 5-10 minutes. Then add 0.38 ml riboflavin-TEMED solution and bring the volume to 10 ml with DI water (0.76 ml for 18 gels). Finally add 5-10 ul persulfate solution (0.24g/ml); mix thoroughly. Riboflavin and APS polymerize quickly under light. *WEAR GLOVES TO TRANSFER TO TUBES; UNPOLYMERIZED ACRYLAMIDE IS A NEUROTOXIN.

C. Preparation of Gels

Close each tube at base with parafilm double-wrapped to seal thoroughly. Mark each one at 12 cm from bottom of tube. Place vertically in the gel tube stand. Draw focus mix into syringe without needle. Attach a 20 gauge syringe needle (6") and carefully add

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mixture to each tube up to mark. Fill from bottom in order to displace bubbles completely. Tap stand to remove any bubbles at the bottom of the tube.

Set up one additional gel to measure pH profile after focusing. (To measure pH profile: After the gels have undergone electrophoresis, remove gel from tube, cut in 1 cm pieces from end, keeping pieces in order, place each 1 cm piece in a small bottle, mince gel as much as possible, add 1 ml-5 ml distilled water to 1 cm pieces. pH on pH meter.)

Allow to polymerize directly under a fluorescent light for 30 minutes then remove 8 M urea and water by blotting with rolled Kimwipes. Overlay with 50 ul DTT-UK_2-NP40. Let polymerize for 2 hours and then blot the tops of the gels to remove the DTT-UK_2-NP40. Add 50 ul UK_2-NP40-DTT to gels.

D. Preparing Tubes

Use thin-walled dialysis tubing cut into 1.5 inch squares. Soak in distilled water. Cut rings of rubber tubing to hold dialysis tubing in place as seals for bottom of tubes. Remove parafilm from tubes with scalpel blade. Electrophoresis apparatus is set up with rubber corks bored to fit tubes snuggly replacing normal rubber grommets as gaskets between upper and lower chambers. Wet tubes with glycerol and slide through corks so they are held firmly. Seal tubes at their base (acid end) with dialysis tubing by placing tubing square under tube and add in 0.2 ml glycerol. Wrap over end of tube to displace all air and fasten with rubber tubing gromments.

E. Loading and Running Sample

Fill lower chamber almost to brim with 0.04 M NaOH. Lower tube rack to immerse lower end of tubes into bottom reservoir. Prepare 0.06 M H_2SO_4 to fill upper reservoir without covering tube tops.

Preparation of sample for tube gels: dissolve lyopholized sample in least amount UK_2-NP40-DTT. Add up to 100 ul of sample mixing well with 50 ul of UK_2-NP40-DTT already there. Carefully fill remaining volume of tube with 0.06 M H_2SO_4 without disturbing. Push tubes down into bottom reservoir until tops are beneath the surface and so that as much of the gel as possible is cooled from the lower chamber. Start coolant running. Reverse connection of terminals to the unit. Set power unit on constant voltage. Run at 75v for 30 minutes to allow stacking. Turn up to 450v for 3.5 hours. Turn off.

F. Removal of Worms

Fill syringe with 0.1% SDS and attach 25 gauge 6 inch needle. Slide needle along gel with bevel facing gel, releasing 0.1% SDS during process until gel slides out onto hand. Air may also be used to gently force out gel.

Place worm gel into a 18 x 150 mm tube with gel straight, without kinks. Place in freezer at -20 C if not used immediately.

G. Equilibration of Worms

Remove top gel sealer from refrigerator and melt in water bath. Add 100 ul B-ME to each worm just prior to use. Remove from freezer, thaw and fill tube with 5 ml of worm equilibration buffer. Rotate at 6 cycles/minute for 10 minutes at room temperature. Drain through a Buchner funnel. Worm is ready to be placed on slab. Pour top gel sealer in slot. Align worm in sealer and add standard piece to one end. Allow sealer to solidify.

H. Preparation of Slabs

Same as in the PROCEDURE FOR 1D PAGE.

I. Running Gel Preparation for Slabs

Same as in the PROCEDURE FOR 1D PAGE except there is no comb so fill sandwich as close to the top as possible and still have room to overlay with 0.1% SDS.

J. Electrophoresis

Same as in the PROCEDURE FOR 1D PAGE except before placing the upper buffer chamber and plates in the lower buffer chamber, seal the worms in with agarose.

K. Removal of Slab Gels

Same as in the PROCEDURE FOR 1D PAGE.

SOLUTIONS USED IN ELECTROPHORESIS

1. Acrylamide - DATD stock
   31.96 g acrylamide
   5.64 g DATD (diallyltartardiamide)
   Bring up to 100 ml with water, filter through Whatman #1, de-gas and store in a dark bottle in refrigerator. Storage life 3 to 4 months.

2. Riboflavin - TEMED
   2.0 mg riboflavin
   0.4 ml TEMED (N,N,N^1,N^1 - tetramethylenediamine)
   Bring up to 50 ml with water. Make fresh each day.

3. UK_2-NP40-Amph (store in 1 ml aliquots in freezer)
   5.5 g 9.3 M urea
   0.5 ml Amph pH 3.5-10
   Bring to 10 ml with 2 % NP40 in 5mM K_2CO_3.

4. UK_2-NP40-DTT (store in 1 ml aliquots in freezer)
   5.5 g 9.3 M urea
   50.0 mg 0.5% DTT
   Bring to 10 ml with 2% NP40 in 5 mM K_2CO_3.

5. Ammonium persulfate (make fresh prior to use)
   For slabs 0.28% 0.28 g APS /100 ml H_2O
   or 0.14 g APS / 50 ml H_2O

6. 0.06 M H_2SO_4 Electrolyte Solution
   2.3 ml conc H_2So_4
   Bring to 1 L with water.

7. 0.04 M NaOH Electrolyte Solution
   2.4 g NaOH
   1.56 L water
   Filter through Whatman #1 and de-gas.

8. Worm Equilibration Buffer
   7.87 g Tris base
   10.0 g SDS
   700.0 ml DI water
   Titrate with 1 M HCL to pH 6.8. Dilute to 1 L. Store in 10 ml aliquots. Immediately prior to use add 100 ul 2-mercaptoethanol to 10 ml aliquot.

9. Running Gel Acrylamide; Bis Buffer A
   150 g acrylamide
   4 g bis acrylamide
   Make up to 500 ml with water. Filter and de-gas, store in dark bottle in refrigerator. Storage life 3-4 months.

10. Running Buffer B (pH 8.8-9)

    90.5 g Tris base
    1.6 ml TEMED
    250.0 ml water

Titrate pH to 8.8 with approximately 120 ml of 1 M HCl. Bring volume to 500 ml with water. Filter solution through Whatman #1, de-gas and store in dark bottle in refrigerator. Storage life 3-4 months.

11. Stacking Gel Buffer E

    29.9 g Tris base
    2.3 ml TEMED

Dissolve in 200 ml water. Titrate the pH to 6.8 with approximately 240 ml of 1 M HCl (1 M HCl = 86 ml conc HCl/L water). Adjust volume to 500 ml. Filter through Whatman #1 paper, de-gas and store in dark bottle. Storage life 3-4 months.

12. Top Gel Sealer

    1.0 g agarose
    100.0 ml worm equilibration buffer w/o B-mercaptoethanol

Warm to 60 C, stirring til agarose dissolves. Add 0.1 ml bromphenol blue. Store in 10 ml aliquots in refrigerator. Add 100 ul B-mercaptoethanol prior to use.

13. SDS - Electrophoresis buffer

    13.5 g Tris base
    64.8 g glycine
    4.5 g SDS

Fill to 4.5 L with water.

14. 20% (w/v) Sodiumdodecyl Sulfate (SDS)

    10 g SDS

Fill to 50 ml with water.

15. 0.1% (w/v) SDS

    5 ml 20% (w/v) SDS
    995 ml water

16. 5 mM Potassium Carbonate (K_2CO_3)

    0.83 g K_2CO_3

Fill to 1 L with water.

17. 10% (v/v) Nonidet-P40 (NP-40)

    10 ml NP-40
    90 ml water

18. 2% (v/v) NP-40 in 5 mM K_2CO_3

    2.0 ml NP-40
    98.0 ml K_2CO_3

19. 8 M urea (Mr urea = 60.06)
    24 g urea
    Fill to 50 ml with water.

20. Preparation of Standards
    A. Prepare standards at a concentration of 2 mg/ml in SDS - dissociation buffer: for 20 ml:

10% (v/v) B-mercaptoethanol (B-ME)	2.0 ml
2% (w/v) SDS	0.4 g
in 10 mM Tris-HCl pH 6.8	18.0 ml

B. Boil standards solution for 5 minutes, store in 1 ml aliquots frozen.

C. Cast standards in agarose using same gel tubes.

D. For each gel use 10.0 mg agarose
7.2 ml SDS buffer
100.0 ul standards solution
Heat to dissolve agarose.

E. Mix and transfer to gel tube sealed at base with parafilm and placed in casting stand.

F. After solution has set, gel is forced out of tubes, placed in petri dish and cut into 1 cm pieces.

G. Standards piece is placed next to worm when running 2D PAGE.

VITA

Allison Elizabeth Henson was born in Somerville, Tennessee on August 5, 1962. She is the youngest of two children born to Christle and Sylvia Longoria Henson. Her brother is Michael Chris Henson. Allison graduated in June 1980 from Briarcrest High School in Memphis, Tennessee. In the fall of 1980, she became an animal science major at the University of Tennessee at Martin. While there, she was active in Alpha Delta Pi national social sorority, the Little Sisters of Alpha Gamma Rho national social and agricultural fraternity, Alpha Zeta national agriculture honorary fraternity and Block and Bridle national animal science organization. Allison earned her Bachelor of Science degree in Agriculture in March 1984 and on April 28, 1984 married another UTM animal science graduate, Rex Wayne Climer II of Bells, Tennessee.

In the summer of 1984, Allison was admitted to the University of Tennessee Department of Animal Science at Knoxville as a graduate student where she was a graduate research assistant. Allison was awarded the Master of Science degree in December of 1986.