BIOCHEMICAL ASPECTS OF

EARLY PREGNANCY IN THE PIG

by

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A thesis submitted to the University of Adelaide
in fulfilment of the requirements for the degree
of Doctor of Philosophy

May, 1985
DEDICATION

This thesis is dedicated to my wife, Judy,
to my children, Kevin and Sam,
and to my parents.
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ABSTRACT

Studies reported in this Thesis examine aspects of early pregnancy in pigs, aimed to identify determinants of the high level of embryonic mortality which occurs prior to implantation in this species.

The first series of experiments describe the content of steroids and free amino acids in the free uterine fluid which constitutes the milieu of blastocysts prior to their implantation at about Day 16 after mating. It was found that several steroid hormones were concentrated in free uterine fluid of both pregnant and non-pregnant animals, in particular pregnenolone sulphate and DHEA sulphate (up to 160-fold increase over plasma levels).

Pregnenolone sulphate was the predominant steroid component of uterine flushings of mated and unmated sows between Days 9 and 15 after oestrus. Compared to plasma values, levels of pregnenolone, DHEA, androstenedione, oestrone and oestrone sulphate were also higher (P<0.05) in free uterine fluid recovered from non-pregnant animals, with further accumulation (P<0.05) of pregnenolone, progesterone, DHEA, testosterone, oestrone and oestradiol in uterine fluids during pregnancy.

The origins of steroid hormones in histotroph were examined in the second group of experiments. The luteal phase sow ovary was shown to be a significant source of pregnenolone, pregnenolone sulphate, progesterone, DHEA, androstenedione, testosterone and oestradiol; progesterone and pregnenolone sulphate being the predominant ovarian products (estimated endogenous production rates of 274 and 85 umol/24h respectively).

Evidence was then adduced to show that the conjugated and unconjugated steroid hormones present in uterine fluid can be metabolized by preimplantation pig embryos (recovered from donors between Days 3 and 6 after mating). Progesterone and pregnenolone were the major secretory products of blastocysts cultured in media supplemented with pregnenolone sulphate (to 1.0 μM), and both steroids were concentrated in blastocoel fluid relative to histotroph (to about 4 and 15 μM respectively). The rate
of secretion of progesterone by blastocysts was depressed in the presence of an inhibitor of 3-β hydroxysteroid dehydrogenase. In view of previous evidence that progesterone can stimulate endometrial secretion and effect immune tolerance of allogeneic implants, it is proposed that accumulation of pregnenolone sulphate in the uterine luminal fluids, and catabolism of this steroid sulphate to C₂₁ steroids by blastocysts, are important biochemical activities during early pregnancy in the sow.

Evidence is presented to show that the total content of 13 of 20 amino acids measured in washings of pregnant uteri exceed (P<0.05) respective non-pregnancy values between Days 9 and 15 after oestrus. This finding is also in accord with the proposal that progesterone of conceptus origin can stimulate endometrial secretory activity during early pregnancy.

High levels of pregnenolone sulphate and DHEA sulphate were also measured in washings of human uteri, suggesting that these sulphoconjugates may be important as precursors to steroidogenesis by blastocysts during early pregnancy in the woman.

Endocrine activity of preimplantation conceptus tissues was also suggested by evidence for release of prostaglandin E₂ by cultured pig blastocysts between Days 4 and 9 after fertilization. Prostaglandin E₂ has previously been implicated in the early recognition of pregnancy, implantation and pregnancy maintenance in sows.

The final series of experiments investigate practical approaches to increasing the efficiency of reproduction in sows. Significant (P<0.01) correlations are shown between litter size at term and levels of oestrone sulphate in plasma of sows between Days 22 and 29 after mating. Measurement of oestrone sulphate in plasma of sows during this period allowed diagnosis of non-pregnancy and prediction of litter size in sows which were pregnant; providing bases to early culling of inefficient sows from pig breeding herds.

The prospect of increasing the efficiency of implantation by presensitizing sows to transplantation antigens is currently being
investigated. Results are reported which indicate substantial gains in litter size (about 2.8 piglets per litter; P<0.05) in sows presensitized to boar antigens by insemination with frozen/thawed spermatozoa at the pre-conceptual oestrus.

Pig embryos used in the in vitro studies in this Thesis (4-8 cell) were collected from slaughtered sows, and over 85% of those embryos developed beyond the blastocyst stage when maintained in an improved culture medium. The final experiment demonstrates the efficacy of this embryo collection and culture technique in an embryo transfer program utilizing slaughtered sows as donors.
DECLARATION

I declare that the experiments reported in this thesis were carried out by myself. Any assistance received from others is specifically acknowledged.

This thesis contains no material which has been accepted for the award of any other degree or diploma in any University and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference is made in the text.

I consent to this thesis being made available for photocopying and loan if accepted for the award of the degree.

Bronte A. Stone
ACKNOWLEDGEMENTS

The experiments detailed in this thesis were carried out in the Department of Obstetrics and Gynaecology, University of Adelaide. I thank Professor L. W. Cox for his permission to work within this Department.

I thank my supervisors, Dr. R. F. Seaman and Dr. P. Quinn, for their encouragement and guidance. Dr. Seaman's inimitable enthusiasm affected all associated with him, providing a stimulating environment in which to work. Dr. Quinn's methodical approach to experimentation in embryology provided me a productive introduction to this research discipline. His tireless indulgence in affairs of conference, and as coryphaeus, consolidated many new and valued friendships. Both Dr. Seaman and Dr. Quinn maintained a keen interest in my research program, and catalysed much constructive discussion on aspects of it. Again, I am particularly grateful to them.

I am indebted to a number of other collaborators. Dr. B. Lloyd, Intensive Industries Pty. Ltd., provided access to mated pigs from which embryos could be collected, and supplied bloods for the study of oestrone sulphate in early pregnancy. Ms. Sylvia Deam, University of Adelaide, was an ever-present source of expert advice on radioimmunoassay methodology. Mr. P. A. Heap, of the South Australian Department of Agriculture's Northfield Pig Research Unit, made sows available for collection of uterine flushings, for the constant infusion studies, and for the ongoing study on effects of uterine presensitization. Dr. O. M. Petrucco, Queen Victoria Hospital, provided human uterine washings for steroid analysis, and collaborated in surgical aspects of the study of ovarian venous outflow. Dr. R. W. Kelly, of the Medical Research Council's Reproductive Biology Unit, Edinburgh, afforded much valuable advice on prostaglandin analysis. Ms. B. M. Godfrey provided statistical assistance. Dr. D. W. Johnson synthesized the deuterated progesterone and determined its occurrence in blood in the constant infusion study. Mr. R. L. Davies, of the South Australian Department of Agriculture, carried out the amino acid analyses. In addition, I thank the routine laboratory staff for their tolerance, and acknowledge the Department of Clinical Photography, Queen Elizabeth Hospital, for their photographic efforts.

I thank all members of the Department of Obstetrics and Gynaecology for their warmth and friendliness during my candidature.

The Australian Pig Industry Research Committee awarded me a postgraduate studentship, and funded the experiments reported herein as part of a larger project on early embryonic mortality in sows. This support is gratefully acknowledged.

Finally, I am indebted to the Director-General of the South Australian Department of Agriculture for granting me study leave for the three years of candidature.
PERSONAL PUBLICATIONS RELATED TO PIG PHYSIOLOGY

(** denotes publications which report experiments detailed in this Thesis)

1. Refereed papers


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3. Thesis

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INTRODUCTION
CHAPTER I

GENERAL INTRODUCTION

1.1 EMBRYONIC MORTALITY AND LITTER SIZE IN THE PIG

Low litter size is a major factor limiting productivity (Paterson, Barker & Lindsay, 1980) and profitability (Bureau of Agricultural Economics, 1972) of pig production enterprises in Australia.

Litter size is a function of ovulation rate, fertilization rate and embryonic mortality and, despite relatively consistent and high ovulation (about 17; Scofield, 1972) and fertilization rates (90 to 95%; Short, 1979), between 15 and 50% of embryos and foetuses reabsorb or are aborted during pregnancy in gilts and in sows (Perry, 1961; Scofield, 1975; Flint, Saunders & Ziecek, 1982). At term, average litters comprise about 10 piglets.

Embryonic reabsorption is a major determinant of litter size at term (Day, Romack & Lasley, 1963) and accounts for about 58% of the total variation in this number in Australian pig herds (Paterson, Barker & Lindsay, 1980).

1.2 THE PATTERN OF EMBRYONIC/FOETAL MORTALITY IN THE SOW

During the first 10 days after mating, 5 to 10% of fertilized ova regress, followed by a loss of 20 to 25% of remaining embryos during the second week of gestation, most of which occurs between Days 8 and 13 post coitum (Scofield, Clegg & Lamming, 1974). A further 10% of embryos die between Days 14 and 25 after mating (Hafez, 1959) and 5-6% of embryos reabsorb between Days 25 and 40 of gestation (Haines, Warnick & Wallace, 1958; Sorensen & Gossett, 1956; Day, Anderson, Emmerson, Hazel & Melampy, 1959).
Further foetal mortality occurs later in pregnancy, particularly near Day 65 (Pomeroy, 1960 a), when placental growth stops and foetal growth progresses rapidly (Pomeroy, 1960 b; Knight, Bazer, Thatcher, Franke & Wallace, 1977). Foetal death may result from placental insufficiency at all stages of pregnancy (Scofield, 1975).

The effects of embryonic/foetal mortality on total numbers of conceptuses during pregnancy in the pig are summarized in Text-figure 1.

1.3 IDENTIFICATION OF AN AREA OF RESEARCH PRIORITY

The practical optimum number of surviving piglets from domestic sows is 14 per litter, as determined by numbers of functional teats during lactation. In view of the high level of peri-natal piglet mortality (between 4 and 8% of all piglets born; Randall, 1972; Sprecher, Leman, Dziuk, Cropper & Dedecker, 1974; Glastonbury, 1976; English & Wilkinson, 1982), litter sizes of 14 or more are thus sought by pig producers, this number being substantially higher than the existing Australian average (near 11; Bureau of Agricultural Economics, 1972).

As the major portion of embryonic loss occurs before Day 25 of pregnancy (Text-figure 1; Hammond, 1921; Corner, 1923; Brambell, 1948; Squiers, Dickerson & Mayer, 1952; Haines, Warnick & Wallace, 1958; Baker, Chapman, Grummer & Casida, 1958; Lerner, Mayer & Lasley, 1958; Hanly, 1961; Perry, 1961; Scofield, Clegg & Lamming, 1974; Flint, Saunders and Ziecik, 1982) and embryonic mortality during the first 25 days of gestation is a significant factor in determining litter size at farrowing (Day et al., 1959), there is a potential for substantial increases in litter size through reducing conceptus losses during the first 25 days of gestation. Average levels of embryonic mortality during this phase of pregnancy in sows are near 30% (Flint, Saunders & Ziecik, 1982), although values reported from different studies vary widely (Table 1).
Text-figure 1: The pattern of embryonic loss in sows between fertilization and term (average +/- range)
Table 1: Levels of embryonic loss in sows during the first 25 days of gestation

<table>
<thead>
<tr>
<th>Reference</th>
<th>Reported embryonic loss (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Robertson, Grummer, Casida &amp; Chapman (1951)</td>
<td>45</td>
</tr>
<tr>
<td>Squiers, Dickerson &amp; Mayer (1952)</td>
<td>35</td>
</tr>
<tr>
<td>Burger (1952)</td>
<td>28</td>
</tr>
<tr>
<td>Perry (1954)</td>
<td>33</td>
</tr>
<tr>
<td>Baker, Self, Chapman, Grummer &amp; Casida (1956)</td>
<td>43</td>
</tr>
<tr>
<td>Sorensen &amp; Gossett (1956)</td>
<td>26</td>
</tr>
<tr>
<td>King &amp; Young (1957)</td>
<td>39</td>
</tr>
<tr>
<td>Lerner, Mayer &amp; Lasley (1957)</td>
<td>34</td>
</tr>
<tr>
<td>Baker, Chapman, Grummer &amp; Casida (1958)</td>
<td>30</td>
</tr>
<tr>
<td>Haines, Warnick &amp; Wallace (1958)</td>
<td>18.3</td>
</tr>
<tr>
<td>Day, Anderson, Emmerson, Hazel &amp; Melampy (1959)</td>
<td>33</td>
</tr>
<tr>
<td>Dyck (1974)</td>
<td>17</td>
</tr>
</tbody>
</table>

In cognizance of the contribution of depressed litter size to the low average reproductivity in pig herds in Australia (Bureau of Agricultural Economics, 1972), the significance of early embryonic mortality as a determinant of litter size in pigs, and the potential for increasing litter sizes through correcting these losses (potential gain of about 30%), this Thesis examines biochemical aspects of interactions between the sow and her conceptuses which may determine embryo survival during early pregnancy.
CHAPTER II

INTERACTIONS BETWEEN THE SOW AND EMBRYOS DURING EARLY PREGNANCY

2.1 INTRODUCTION

Embryonic tissues are parasitic, their maintenance being dependent upon maternal support in all viviparous species. Endometrial secretions must then meet the changing nutrient demands of embryonic tissues, and embryonic tissues must have the capacity to absorb and incorporate these nutrients. Parallel and ordered morphological development of embryonic and uterine tissues is thus a prerequisite to the establishment of a viable symbiotic relationship between these tissues in pregnancy. Lower conception rates and higher embryonic mortality following asynchronous embryo transfers in sows (e.g. Poige, 1982) provide circumstantial evidence which corroborates the consequence of asynchronous development of these tissues in the sow.

Factors which induce asynchrony in development of the major components of the embryo/endometrial complex during early pregnancy can thus compromise successful implantation. In this regard, and as a preface to the studies in this Thesis which examine aspects of early embryonic development, the following section outlines morphological changes in the embryo/endometrial complex during the preimplantation interval of pregnancy in sows. Changes in embryo morphology between Day 3 and 16 after fertilization are shown in Text-figure 2.
Text-figure 2: Morphological changes of pig embryos during the first 16 days of gestation

A. Pig morulae collected about 3 days after mating/fertilization

B. Blastocysts at about 4 days after fertilization

C. Blastocysts at about Day 5 after fertilization

D. Hatching of blastocysts from zonae pellucida, commencing about 6 days after fertilization

E. Dumb-bell appearance characteristic of part-hatched blastocysts (about 6 days after fertilization)

F. Hatched blastocyst 7-8 days after fertilization

G. Flaccid, wrinkled trophoderm; characteristic of blastocysts about 11 days after fertilization

H. Proliferating trophoderm, about 12 days gestation

I. Filamentous blastocysts, 13 days post coitum

J. Foetal membranes and a pig embryo recovered from a sow 16 days after mating
2.2 DEVELOPMENT OF THE EMBRYO/ENDOMETRIAL COMPLEX

Fertilization to blastocyst formation

Pig embryos remain in the fallopian tubes for 46-48 h after the commencement of fertilization, undergoing two mitotic divisions before entering the uterus at the 4-cell stage (Hunter, 1977). During this time, metabolism of embryos is influenced by the nature and volume of tubal secretions, these functions being dependent upon appropriate endocrine stimulation of the tubal endothelium (ibid). The ampullary segment of the fallopian tube exhibits greater secretory activity than the isthmus and, biochemically, is a more favourable environment for embryos retained in the tubes by artificial means. In this respect, pig embryos restricted to the ampulla can develop to the blastocyst stage (Pope & Day, 1972) whereas embryos restricted to the isthmus regress (Murray, Bazer, Rundell, Vincent, Wallace & Warnick, 1971).

As embryos descend the isthmus of the fallopian tubes, the outer portion of the zona is still permeable to competent spermatozoa and, in regions of progressively higher sperm density, more sperm attach to the zona, but do not penetrate (Hancock, 1961). The number of spermatozoa embedded in the zona pellucida by the 4-cell stage may exceed 200 (Hunter & Leglise, 1971; Hunter, 1974).

The embryonic block to polyspermy remains effective until at least the time of entry of embryos into the uterus, and the zona retains this property until it is shed on about Day 6 after fertilization (see Text-figure 2; Hunter, 1977).

Precise timing of entry of embryos into the uterus is critical for embryonic survival (Chang, 1950), but few management practices are likely to perturb this aspect of tubal transport, with the possible exception of administration of progesterone (Day & Polge, 1968; Hunter, 1972) or synthetic progestagens (Dziuk & Polge, 1962).
Growth of the blastocyst

Following hatching from the zona pellucida (Day 6-8 post coitum; Text-figure 2), the trophoblast proliferates rapidly (Heuser & Streeter, 1929; Green & Winters, 1946). By Day 10 of gestation, blastocysts enlarge to wrinkled spheres, about 2 mm in diameter. One day later, blastocysts appear as flaccid sacs, about 5 mm in diameter (Text-figure 2).

During the ensuing 2-3 days, blastocysts undergo rapid elongation to form tubular, bilaminar, thread-like structures about 1.2 m in length (see Text-figure 2; Perry & Rowlands, 1962; Anderson, 1978). Cellular hypertrophy and remodelling in endoderm and trophoderm layers (Geisert, Brookbank, Roberts & Bazer, 1982; Geisert, Renegar, Thatcher, Roberts & Bazer, 1982), and an exponential increase in blastocyst protein content (Wright, Grammer, Bondioli, Kuzan & Menino, 1983), are associated with this proliferation. Intra-uterine displacement and mixing of pig embryos between uterine horns occurs predominantly during this same period (Days 9 to 11 after mating; Dziuk, Polge & Rowson, 1964; Dhindsa, Dziuk & Norton, 1967; Polge & Dziuk, 1970). Blastocyst migration results in even spacing of embryos prior to the onset of implantation, irrespective of the number of corpora lutea in each ovary. At the commencement of implantation, trophoblast tissue is in contact with the mesometrial surface of the endometrium over the entire length of both uterine horns, and failure to establish this contact in both uterine horns leads to pregnancy failure (du Mesnil du Buisson, 1961).
Implantation

Tenuous attachment of the trophoblast to the endometrium commences as short microvilli extend from the trophoblast surface, coinciding with the growth of microvilli on the endometrial surface (Crombie, 1970). Microvilli appear at about Day 13 of gestation, followed about one day later by points of loose attachment between trophoblast and endometrium (ibid). Intimate attachment between embryonic and maternal epithelia by interlocking villi is established by about Day 18 after mating (Crombie, 1970). The trophoblast does not further invade endometrial tissue during pregnancy in the sow, a property of the diffuse epitheliochorial placentation in this species (Perry, 1981). The ultrastructure of the trophoblast of the pig placenta has been described recently (Bielanska-Osuchowska, 1979).

During implantation, folds of the proliferated trophectoderm overlay furrows in the endometrium (Amoroso, 1952), the summits and sides of the trophectoderm ridges being the anatomical sites of exchange of gases and other diffusible substances between the embryo and endometrium (Wislocki & Dempsey, 1946). In these regions, uterine and allantoic capillaries indent the respective epithelia (Goldstein, 1926), thereby reducing the length of the diffusion pathway.

Principal sites of histotrophic nutrition are the areolae. Each areola consists of a trophoblastic and a uterine part, between which lies a cavity containing uterine 'milk', an amorphous secretion from the uterine epithelium (Dantzer, Bjorkman & Hasselager, 1981) which contains detritus from the degeneration of endometrial epithelial cells (Amoroso, 1952).

Through the diffuse, non-invasive, epitheliochorial placenta, the pig embryo derives nutrients from either the circulating maternal blood (haemotroph) or from secretory products of the endometrium (histotroph). Many endometrial products, including uterine specific acid phosphatase, are synthesized by the uterine endometrial glands, and are transported via the placental areolae into the allantoic fluid (Bazer, Chen, Knight,
Schlosnagle, Baldwin & Roberts, 1975; Chen, Bazer, Gebhardt & Roberts, 1975). The placental areolae also serve as specific sites for absorption and transport of secretions of the uterine glands (haemotroph and histotroph; Goldstein, 1926; Brambell, 1933). These secretions are the likely source of the allantoic fluid proteins (Bazer et al., 1975).

In these ways, proliferation of the trophectoderm as a nutrient-absorbing surface is prerequisite to effective nutrient exchange between the embryo and mother during embryonic growth and development (Fazleabas, Bazer & Roberts, 1982; Geisert et al., 1982 b). Embryos which fail to elongate are then assumed to be at a competitive disadvantage prior to, and at, implantation.
2.3 THE INTRAUTERINE MILIEU

2.3.1 Introduction

During the exponential growth phase prior to implantation, viable embryos are dependent upon provision of nutrients from histotroph, this nutrient pool being the product of endometrial secretion by exocytotic disgorgement, and of transport of nutrients across endometrial membranes. Rates of uterine secretion begin to increase about two days before ovulation, being highest on about Day 2 after the onset of oestrus (Iritani, Sato & Nishikawa, 1974). Quantitatively, rates of uterine fluid secretion vary widely about average values of 7 ml/24 h (during oestrus) and 3 ml/24 h (during dioestrus; Iritani, Sato & Nishikawa, 1974; Engle, Dunn, Hood, Williams, Foley & Trout, 1968). In pregnant animals, endometrial tissues overlain by conceptus tissues show a higher secretory activity than unoccupied areas of gravid uteri (Basha, Bazer & Roberts, 1980).

Following implantation (near Day 16 after fertilization; Perry, 1981), nutrients in histotroph and in haemotroph nourish conceptus tissues.

Deficiencies in quantitative and/or qualitative properties of endometrial secretions during the first 25 days of pregnancy will impose constraints on embryonic growth which may lead to the demise of the growth retarded conceptuses at that time, or during a later phase of pregnancy. In this respect, identification of nutrients within endometrial secretions can indicate which elements of histotroph are essential to embryonic growth in vivo. The occurrence of potential anabolic elements in histotroph of sows is summarized below.

2.3.2 Composition of histotroph

Protein. Proteins in uterine fluid can act during early pregnancy as enzymes, carrier molecules and regulators of genetic activity (histone and
nonhistone chromosomal proteins).

The rate of protein secretion by the endometrium of the pig increases during the luteal phase of the oestrous cycle (Murray, Bazer, Wallace & Warnick, 1972; Squire, Bazer & Murray, 1972; Iritani, Sato & Nishikawa, 1974) and during early pregnancy (Zavy, Bazer, Thatcher & Wilcox, 1980), attaining a maximum level on about Day 15 of pregnancy (Murray et al., 1972; Bazer, 1975). Rates of secretion of protein by the endometrium of unmated sows are quantitatively correlated with plasma progesterone concentrations (Murray et al., 1972).

Concomitant with the increase in rate of protein secretion is a change in protein composition of the secretion. For example, qualitative analysis of the proteins present in the endometrial secretions reveals at least seven non-serum proteins in histotroph between Days 14 and 16 of the oestrous cycle, these proteins being absent from uterine secretions collected before Day 12 after the onset of oestrus (Squire, Bazer & Murray, 1972).

A major component of endometrial protein secretion comprises a uterine-specific, purple, acid phosphatase (MW about 32000 daltons; Squire, Bazer & Murray, 1972; Schlosnagle, Bazer, Tsibris & Roberts, 1974) to which is bound one ferric ion/molecule (Chen, Bazer, Cetorelli, Pollard & Roberts, 1973; Schlosnagle, Sanders, Bazer & Roberts, 1976). This protein (uteroferrin) has been purified (Chen et al., 1973) and characterized (Bazer, 1975). Uteroferrin activity (measured as acid phosphatase) in histotroph of pregnant and non-pregnant gilts is maximal about 15 days after oestrus (Chen et al., 1975; Zavy, Roberts & Bazer, 1984).

The pattern of activity of aminoacylpeptidase, a less predominant protein in uterine fluids of pigs, differs from that of acid phosphatase (Zavy, Roberts & Bazer, 1984), although aminoacylpeptidase and acid phosphatase activities are both induced by progesterone (Roberts, Bazer, Baldwin & Pollard, 1976).

Acid phosphatase, aminoacylpeptidase and lysozyme activities continue to accumulate in allantoic fluid of pigs beyond Day 30 of pregnancy (Bazer et
al., 1975; Roberts et al., 1976) and are released by explants of porcine endometrium cultured in vitro (Basha, Bazer & Roberts, 1979).

A retinol-binding protein (MW <17000 daltons) identified in secretions of the endometrium during the luteal phase of the cycle (Adams, Bazer & Roberts, 1981) is estimated to comprise about 5% of the total fraction of low molecular weight proteins induced by progesterone, and has been ascribed a role in the transport of water-insoluble nutrients from the endometrium to the conceptus (ibid).

Roles for other biologically active proteins which have been identified in porcine uterine secretions (e.g. cathepsins Al, Bl, C and D; Bazer, 1975) during the first 25 days of embryonic growth have not yet been defined (Aitken, 1979). However, a large number of peptides can be synthesized by endometrial tissues of other mammals (about 800 in the rat; Lejuene, Lecocq, Lamy & Leroy, 1982), and numerous and diverse roles have been ascribed to these molecules in early pregnancy.

Secretion of proteins by the endometrium of sows is induced by treatment with progesterone or progesterone plus oestradiol, but not with oestradiol alone (Knight, Bazer & Wallace, 1973; Roberts et al., 1976; Basha, Bazer & Roberts, 1980). However, the total content of protein in uterine flushings collected from mated and un-mated gilts between Days 10 and 14 after oestrus changes in parallel with total oestradiol content of the flushings (Geisert et al., 1982 a) and is not directly related to progesterone content. These results support the earlier suggestions that progestagens and oestrogens act synergistically in the control of secretion of macromolecules by the porcine endometrium, and are in accord with results from studies with other mammals in early pregnancy (e.g. Fishel, 1979).

In regard to endometrial protein synthesis, studies on rates of $[^3]$H-leucine incorporation into endometrial homogenates in vitro have indicated decreasing rates of protein synthesis by endometrial explants between Days 10 and 16 after oestrus in pregnant and in non-pregnant sows (Guthrie, 1982). In this same study, protein synthesis continued to decline
from Day 16 to 19 after mating in the pregnant gilts, but increased significantly between Days 16 and 19 after oestrus in unmated animals. Reasons why endometrial protein synthesis should be suppressed during the phase of stimulated endometrial protein secretion in pregnant gilts have not yet been proposed.

Amino acids. A protein source is essential to growth of mammalian blastocysts (Gwatkin & Meckley, 1965) and developmental responses to protein have been attributed to effects of amino acids contributed by the protein source (Brinster, 1972), including the provision of free amino acids which enhance protein synthesis (Sellens, Stein & Sherman, 1981) and stimulate expansion of mammalian blastocysts in vitro (Daniel & Krishnan, 1967). As amino acids and other substrates for protein anabolism by pre-implantation blastocysts are provided from endometrial secretions, levels of amino acids in histotroph collected from early pregnant sows may reflect peculiarities in substrate requirements for elongation of porcine blastocysts in vivo. However, levels of amino acids in histotroph of pregnant sows have not been reported, and only two studies (Engle et al., 1968; Iritani, Sato & Nishikawa, 1974) have documented the amino acid composition of histotroph collected from nonpregnant, sexually mature, sows. Unfortunately, Engle et al.'s results were reported in abstract form only, and levels of amino acids in the uterine fluids were not stated. The results of the latter study are summarized below.
Table 2: Content of total free amino acids in histotroph collected from un-mated sows (from Iritani, Sato & Nishikawa, 1974; mM)

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Stage of the oestrous cycle</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Oestrus #</td>
</tr>
<tr>
<td>Alanine</td>
<td>0.28</td>
</tr>
<tr>
<td>Arginine</td>
<td>0.06</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>0.03</td>
</tr>
<tr>
<td>Cystine</td>
<td>0.01</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>0.21</td>
</tr>
<tr>
<td>Glycine</td>
<td>0.40</td>
</tr>
<tr>
<td>Histidine</td>
<td>0.07</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>0.12</td>
</tr>
<tr>
<td>Leucine</td>
<td>0.25</td>
</tr>
<tr>
<td>Lysine</td>
<td>0.23</td>
</tr>
<tr>
<td>Methionine</td>
<td>0.02</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>0.09</td>
</tr>
<tr>
<td>Proline</td>
<td>0.01</td>
</tr>
<tr>
<td>Serine</td>
<td>0.08</td>
</tr>
<tr>
<td>Threonine</td>
<td>0.06</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>0.08</td>
</tr>
<tr>
<td>Valine</td>
<td>0.37</td>
</tr>
</tbody>
</table>

# Between Days 0 and 4 after the onset of behavioural oestrus

The total free amino acid content of histotroph collected from dioestrous sows (Days 5-11 after the onset of oestrus) is about triple the respective value for oestrous (Days 0-4 after the onset of oestrus) fluid (7.46 and 2.37 mM respectively; Iritani, Sato & Nishikawa, 1974). Both Iritani et al.'s and Engle et al.'s studies identified glycine, alanine and lysine as predominant amino acids in histotroph of the sow. Iritani et al. also suggested that cyclic changes in the concentration of amino acids in histotroph may reflect changing demands for amino acids by gametes. This proposal has not yet been substantiated.

Specific amino acid requirements for expansion of blastocysts in vitro have been defined (Kane & Poote, 1970; Daniel & Olson, 1968; Weitlanf & Greenwald, 1967), and are discussed later in Chapter 3.3.
Steroid hormones. The total content of oestradiol in uterine flushings collected from early pregnant gilts increases about six-fold between Days 10 and 12 after mating (Zavy et al., 1980; Bazer, Geisert, Thatcher & Roberts, 1982; Geisert et al., 1982 a), then declines by Day 14 (Bazer et al., 1982; Geisert et al., 1982 a). The pattern for total oestrone is similar, the highest values appearing for flushings of uteri containing filamentous blastocysts (ibid.). These same authors observed a concomitant increase in oestrone sulphate and oestradiol sulphate content of flushings containing tubular and filamentous blastocysts at Day 12 of gestation. This rise could be attributable to endometrial sulfoconjugation (Pack & Brooks, 1974) of ovarian follicular oestrogens, the secretion of which increases during this period (Hansel, Concannon & Lukaszewska, 1973). Alternatively, the oestrogen substrate may derive from conceptus tissues (see Chapter 2.5.3.1). Either mechanism is consistent with significant increases in oestrone sulphate concentrations in utero-ovarian venous plasma between Days 10 and 12 after coitus (Stoner, Geisert, Bazer & Thatcher, 1981).

Vitamins. A yellow, dialysable, hydrophilic chromophore is present in the uterine lumen of pigs on Day 8 after oestrus (Moffatt, Murray, Grifo, Haynes, Kinder & Wilson, 1980). The chromophore appears to be riboflavin, and is present in the lumen at a total level near 100 µg/uterus (ibid.).

Minerals. Calcium is detectable in flushings of uteri of early-pregnant and of non-pregnant pigs (Geisert et al., 1982 a). Changes in the total content of calcium in uterine flushings parallel changes in total protein content, with calcium levels increasing up to Day 12 after mating, then decreasing to Day 14. Total levels of calcium in flushings of non-pregnant uteri are lower than pregnancy levels, and are stable between Days 10 and 14 after oestrus (ibid). In regard to possible effects of pregnancy on release of calcium by the endometrium, conceptus tissues synthesize oestrogens (see
Chapter 2.5.3.1) which can influence the movement of calcium in endometrial cells of the rat (Pietras & Szego, 1975). Calcium can then effect exocytosis of secretory vesicles from the endometrium, as it does from cells of the adrenal medulla and cortex, salivary gland, pancreas, kidney and nerve cells (Rasmussen & Goodman, 1977; Rubin & Laychock, 1978). This mechanism is consistent with parallel changes in the uterine content of oestrogens, calcium and total protein during early pregnancy in the pig (Geisert et al., 1982 a).

Prostaglandins. Calcium released by the endometrium may activate phospholipase A2 in the endometrium (or in conceptus tissues) to enhance prostaglandin (PG) synthesis in these tissues (Rubin & Laychock, 1978). In this respect, the total content of PGs E₂ and F₂ in washings of pregnant uteri of pigs exceed non-pregnancy values and increase between Days 10 and 14 of gestation, during which time levels of calcium in pig uteri are also increasing (Geisert et al., 1982 a). An oestrogen and calcium-induced mechanism for accumulation of PGs in histotroph during early pregnancy in pigs is more plausible than an alternative proposal that endometrial prostaglandin secretions are 'redirected' away from the uterine vein, toward the uterine lumen, during early pregnancy (e.g. Frank, Bazer, Thatcher & Wilcox, 1977; 1978).

Levels of PGE in histotroph collected from non-pregnant sows (Days 10 to 14 after oestrus) are quantitatively similar to PGF values, but exceed PGF by about five times during the equivalent phase of pregnancy (Geisert et al., 1982 a).

Roles for PGs in embryo/maternal interactions during early pregnancy in the sow are discussed in more detail in Chapter 6.
2.4 DETERMINANTS OF EMBRYONIC SURVIVAL DURING THE FIRST 25 DAYS OF GESTATION IN THE SOW

2.4.1 Introduction

Many internal and external environmental factors are known to influence embryonic survival and the outcome of early pregnancy. Studies in this Thesis address factors associated with high levels of early embryonic mortality in pigs, but do not attempt to re-examine effects of all embryotoxic factors on the outcome of early pregnancy in this species. Those environmental factors which depress embryonic survival in pigs must, however, be taken into account when investigations of early pregnancy are undertaken. This Thesis describes such studies. Therefore, the more significant of the physiological and environmental factors which can affect the outcome of early pregnancy in sows are outlined below.

2.4.2 Time of entry of embryos into the uterus

Following fertilization, pig embryos remain at the ampullary-isthmic junction for about two days before traversing the isthmus, into the uterus (Thibault, 1972). Premature entry of embryos into the uterus is detrimental to embryo survival (Moore & Shelton, 1964; Day & Polge, 1968), as is delayed transport (Murray et al., 1971).

2.4.3 Intra-uterine crowding and position of embryos

Intra-uterine crowding is associated with increased embryonic mortality in pigs (Hammond, 1921; Perry, 1954; Rathnasabapathy, Lasley & Mayer, 1956; King & Young, 1957; Hunter, 1966; Dziuk, 1968; Weibel & Dziuk, 1974; Knight et al., 1977). Furthermore, treatments which result in intra-uterine crowding result in a high incidence of embryonic mortality, although this
occurs predominantly after Day 30 of pregnancy (Hunter, 1966; Webel & Dziuk, 1974; Knight et al., 1977). These observations, together with evidence that embryonic survival is higher in sows whose uteri have elongated in response to progesterone/oestrone treatment (Reddy, Mayer & Lasley, 1958), imply that a critical area of endometrial surface must be available to each embryo for effective placentation and embryonic survival (Wraithall, 1971).

Waldorf, Foote, Self, Chapman & Casida (1957) and Perry & Rowell (1969) have shown that pig embryos at the cervical and ovarian extremities of uterine horns are larger than intermediate embryos and suggested that the larger embryos were more viable. In their study on foetal size and spacing in domestic and feral pigs, Hagen, Kephart & Wagness (1980) could not demonstrate a consistent relationship between foetal weight and the amount of uterine space available to each foetus, as indicated by the distance between adjacent foetuses.

2.4.4 Nutrition

Numerous nutritional approaches to intra-uterine growth retardation and foetal malnutrition in the human have been reported (e.g. Bustamante, 1980). These studies implicated maternal malnutrition as one of the most common factors associated with human foetal malnutrition, and parallel studies with laboratory animals have associated maternal malnutrition with foetal resorption (e.g. Pennycuik, 1964).

In their study with the pig, Robertson et al. (1951 a) examined the influence of high and low protein levels in feed (15 and 11% crude protein), and of restricted and ad libitum feeding, on embryonic death. Levels of embryonic survival were not affected by the protein level in the ration, but there was a higher degree of embryonic survival in those sows fed according to a restricted feeding regime (67% survival compared to 43% survival in sows fed ad libitum). Similarly, Dutt & Chaney (1968) showed increased embryonic survival when feed intake was reduced (from 4.1 to 1.25 kg/day).
after Day 10 of pregnancy, with a larger increase when feed intake was restricted from the day of mating. Dyck & Strain (1980) reported increased embryonic survival in gilts at Day 35 of gestation (75.8 to 86.7%) when the level of feed intake was reduced from 2.5 to 1.5 kg/day, consistent with results of the earlier studies which had shown that a high total digestible nutrient intake by gilts from approximately 90 days of age until 25 days of gestation was harmful to embryo survival (Christian & Nofziger, 1952; Self, Grummer & Casida, 1955). The association between low level feeding and higher embryo survival during the first 25 days of gestation appears to apply during pregnancy in gilts (Self, Grummer & Casida, 1955) and in sows (Tribble, Pfander, Lasley, Zobrisky & Brady, 1956).

Levels of embryonic survival at Day 25 after mating also appear to be higher in gilts fed high-fibre rations (inclusion of corn cobs at 40% of the ration on a weight basis) compared with control sows fed a low fibre diet (71.5 and 54.5% embryonic survival respectively; Spies, Zimmerman, Self & Casida, 1959).

In contrast to the above findings, Dyck, Palmer & Simaraks (1980) fed gilts at three different levels (1.50, 2.25 and 3.00 kg/day) throughout pregnancy and observed no effect of feeding level on the number of corpora lutea, number of foetuses, foetal survival or mean foetal weight. Similarly, Haines, Warnick & Wallace (1958) and Haines, Warnick, Wallace & Edwards (1957) could not show a change in embryonic survival in sows fed limited intakes at the time of mating.

After Day 25 of pregnancy, foetal mortality can increase in response to restricted feeding of sows (Waldorf et al., 1957; Gossett & Sorensen, 1959), and recent evidence indicates that rates of weight gain by sows during gestation are positively associated with litter size at term (Bereskin & Frobish, 1981). Results from the latter study did not include corpus luteum (CL) numbers, so could not indicate whether larger litter sizes from faster-growing pregnant sows resulted from reduced embryonic mortality, or from higher ovulation/fertilization rates.
Studies with other polytocous mammals have demonstrated increased embryonic mortality in females which are fasted during early pregnancy (McClure, 1959, 1961a).

In respect to the evidence for reduced embryonic survival associated with reduced feed intake, the stress of reduced feed intake may induce embryonic reabsorption by stimulating hypersecretion of corticosterone (Robson & Sharaf, 1952; Macfarlane, Pennycuick & Thrift, 1957). Alternatively, chronic inanition may result in anoestrum or terminated pregnancy through impaired gonadotrophin release by the hypophysis (Werner, 1939; Pomerantz & Mullinos, 1939; Rinaldini, 1949). Evidence that pregnancy in fasted rats can be extended by daily treatment with progesterone and chorionic gonadotrophin (McClure, 1961b), yet is not maintained in fasted rats which were adrenalectomized and treated with desoxycorticosterone acetate (McClure, 1961b), suggests that the pathogenesis of embryonic mortality caused by fasting involves a failure of hypophysial gonadotrophic function and is not associated with hypersecretion of corticosterone.

Failure by investigators to adequately define dietary characteristics frustrates synthesis of the discrepant results presented above. However, in regard to practical recommendations for feeding early pregnant sows, either fasting or over-feeding during early pregnancy will be expected to be associated with reduced embryonic survival. In commercial practice, few sows would be fasted at this time, and the restricted level of feeding which is suggested above would provide a digestible energy (DE) intake of about 22 MJ/day, which would meet the maintenance energy requirement of weaned sows of about 120 kg liveweight. However, a large proportion of sows are fed at high levels (to about 4 kg/day) during the first 2 to 4 weeks of gestation in an attempt to restore body condition lost during lactation. In these cases, mating could be delayed for one oestrous cycle, during which time the weaned animals could be pre-conditioned for a restricted feeding routine (to a maintenance level of about 1.5 to 2.0 kg/day; feed DE level near 14 MJ/kg) during the first 3-4 weeks of pregnancy. This regime is consistent with
high embryonic survival. In addition, as ovulation rates are higher at the second oestrus after weaning, litter sizes can be increased by up to two piglets if mating of weaned sows is delayed and mated sows are fed according to the restricted regime detailed herein.

2.4.5 Environmental strain

The pig is a homeotherm, susceptible to cold at birth and susceptible to heat at maturity. The thermoneutral zone of mature domestic pigs extends from about 21°C (Capstick and Wood, 1922) to about 28°C (Stone, 1982). The high thermal insulation provided by a poorly vascularized and thick (about 17 mm) fat covering, the low critical temperature, and an inability to sweat effectively (Ingram, 1965), results in positive heat balance in pigs housed under a range of conditions during Mediterranean summers (Stone & Heap, 1982), reflected in increased core temperature of adult animals at air temperatures above 30°C (Heitman & Hughes, 1949; Stone, 1982).

Exposure of mature pigs to thermal stress is also associated with increased plasma levels of adrenocorticotropic hormone (ACTH) and adrenal steroids (Marple, Jones, Alliston & Forrest, 1974; Kovalenko, Akulov, Pesenko & Bondarenko, 1975; Lundstrom, Bosu & Gahne, 1975), indicating stimulation of the pituitary-adrenal axis. Plasma corticosteroid levels in pigs in these studies rose to near 280 nm, compared with basal levels near 11 nm (Stone, 1984). In view of the protein catabolic effects of corticosteroids, increased activity of the hypothalamic-pituitary-adrenal axis during heating of sows during early pregnancy may initiate increased embryonic and foetal reabsorption.

Similarly, hyperthermia of the gravid uterus during heating of sows may itself affect development of embryos. In this respect, there is a large body of data which describes metabolic inefficiencies associated with depressed enzyme activity at temperatures divergent from optima and, in most
mammalian tissues studied, optimum temperatures are near normal body temperature. It is assumed that embryonic tissues of pigs during the first 25 days of pregnancy are poikilothermic and that the metabolic efficiencies of embryonic tissues will be dependent upon homeothermy in the host uterus.

Changes in embryonic survival in sows in response to heating during early pregnancy have been reported. For example, in 1957, Whatley, Palmer, Chambers & Stephens observed diminished litter sizes in pregnant sows maintained at 35°C during the first trimester of pregnancy, compared with control sows housed in evaporatively cooled (about 25°C) sheds (average litter sizes of 7.7 and 10.0 respectively). More recent studies by Edwards, Omtvedt, Turman, Stephens & Mahoney (1968) and by Omtvedt, Nelson, Edwards, Stephens & Turman (1971) have shown that heat stress of gilts during the first 16 days after mating can result in lower conception rates and increased embryonic reabsorption in pregnant animals, leading to smaller numbers of foetuses at Day 30 post coitum.

In regard to potential indirect effects of heat on the efficiency of early pregnancy, via perturbed endocrine function, heating gilts during the first 8 days of pregnancy effects elevated progesterone and depressed oestradiol levels in maternal plasma (Kreider, Wettemann, Johnson & Turman, 1978). These steroids affect embryo migration (Pope, Maurer & Stormshak, 1982), immune cytoprotection of the embryo (Siiteri, Febres, Clemens, Chang, Gondos & Stites, 1977), endometrial secretory activity (Knight, Bazer & Wallace, 1973; Schlosnagle, Sanders, Bazer & Roberts, 1975; Roberts et al., 1976; Adams, Bazer & Roberts, 1981; Fazleabas, Bazer & Roberts, 1982), and embryonic survival during the first 25 days of gestation (Tillson & Erb, 1970; Wildt, Culver, Morcom & Dukelow, 1976; Flint, 1984; see Chapter 2.5.2). Changes in the progestagen and oestrogen status of early pregnant sows may thus induce a proportion of the embryonic mortality which occurs in sows exposed to air temperatures above 29°C (Kreider et al., 1978; Wettemann, Bazer, Thatcher & Hoagland, 1984).

Heat-induced suppression of embryonic growth in pigs (as indicated by...
decreased weight and $^3$H-leucine incorporation into macro-molecules; Wettemann et al., 1984) does not appear to be associated with changes in flow, $pO_2$ or $pCO_2$ of blood in the uterine artery (Wettemann, Bazer, Caton, Roberts & Thatcher, 1982).

Heat strain also suppresses embryonic development during early pregnancy in other mammalian species. For example, heat stress retards foetal growth in sheep (Hopkins, Nolan & Pepper, 1980), stimulates reabsorption of rat foetuses (Macfarlane, Pennycuik & Thrift, 1957), and may reduce foetal viability in the human (Macfarlane, 1963).

2.4.6 Genetic factors

**Chromosomal abnormalities.** Death of conceptuses exhibiting chromosomal variants is consistent with early elimination of mutants from the gene pool and is thus a necessary component of selection (Bishop, 1964). Variants which are excluded include those genetic traits which lead to increased embryonic death through inheritance of homozygous recessive genes encoding lethal characteristics (see Bishop, 1964), including sticky anaphase, arrested embryogenesis and autosomal translocation; all of which are associated with increased embryonic mortality in the pig (Bouters, Bonte & Vandeplasche, 1974). A further component of embryonic loss is associated with induced structural chromosomal aberrations in conceptuses, such as those which allow polyspermic fertilization (e.g. mating late during the oestrous cycle: Austin & Braden, 1954; Hunter, 1967a, b; Day & Polge, 1968; progesterone treatment before mating: Polge & Dziuk, 1965; Day & Polge, 1968).

Genes of the major histocompatibility complex (MHC; as defined for pigs by Gotze, 1977) may also influence early embryonic mortality (Goldbard & Warner, 1982; Warner, Gollnick & Goldbard, 1984), so mutations of MHC genes may be associated with increased levels of embryonic death during the first 25 days of pregnancy.
In general, chromosomal abnormalities in pig blastocysts are triploid or tetraploid (McPeel, 1967), and Moon, Rashad & Mi (1975) have implied a high incidence of these polyploid variants in pig blastocyst tissues (in 4/15 Day 11 blastocysts karyotyped). However, when inner cell masses and trophoblast tissues from individual preimplantation pig blastocysts were cytogenetically examined (e.g. Long & Williams, 1982), polyploid cells occurred primarily in the trophoblast (about 50% of all polyploid and mixoploid cells). The incidence of true mixoploidy in pig embryos is very low (<11%; ibid).

Average mortality rates of preimplantation pig embryos are near 30% (Table 1), so karyotypic variants can account for a maximum of about one third of all embryonic loss during the first 25 days of gestation. This proportion agrees closely with an estimate for early pregnancy in women (about 30%; Nishimura, 1970).

Boar. Smidt (1962) reported a direct association between levels of embryonic mortality and sires of the conceptuses. High rates of embryonic loss also characterize pregnancies to particular boars (Rathnasabapathy, Lasley & Mayer, 1956; Reddy, Lasley & Mayer, 1958; Perry, 1960; Perry & Rowlands, 1962), consistent with heritability of a genetic trait which predisposes to a high level of embryonic mortality.

Boars vary significantly with respect to implantation rate of zygotes fertilized by their gametes, and to the size of litters sired (Swierstra & Dyck, 1976). Furthermore, significant positive correlations between implantation rates and average litter sizes sired by particular boars (Swierstra & Dyck, 1976) suggest that litter size in pig herds can be effectively increased by culling boars whose offspring show low implantation rates.

Breed. Robertson, Grummer, Casida & Chapman (1951) reported higher rates of embryonic survival in Poland China compared with Chester White breeds of gilts; and Short, Zimmerman & Sumption (1963) reported increased embryonic
survival in Duroc compared to Yorkshire (Large White) gilts. Other groups (Robertson et al., 1951 a; Self, Grummer & Casida, 1955; Baker et al., 1958) have failed to identify relationships between breeds of sires and/or dams, and the survival of their embryos.

Inbreeding. Litter size is usually depressed in inbred sows (Sellier, 1970; Mikami, Fredeen & Sather, 1977; Dickerson, Blunn, Chapman, Kottman, Kridor, Warwick & Whatley, 1954) and is determined by levels of inbreeding in both the dam and litter (Dickerson et al., 1954). This attribute of inbreeding is consistent with evidence for higher rates of embryonic loss in inbred strains of pigs, losses which can be corrected by out-crossing (Squiers, Dickerson & Mayer, 1952; Rampacek, Robison & Ulberg, 1975; Flint, Saunders & Ziecik, 1982).

2.4.7 Immunological factors

Presensitization of females against paternal tissue antigens of future conceptuses can result in significantly larger litters than those produced by control females (mouse- James, 1965; rat- Beer & Billingham, 1974; hamster- Beer, Scott & Billingham, 1975). Beer and Billingham (1974) have shown that prior local sensitization of one uterine horn of the rat improves the subsequent reproductive performance of that horn compared with that of the contra-lateral, non-immunized horn (in terms of the number and weight of the foeto-placental units which implant and develop to term) following mating with males against whose tissue allo-antigens their sensitivity is directed.

In regard to increasing the efficiency of implantation in pigs by hypersensitizing sows to boar lymphocyte antigens, a preliminary attempt to describe a uterine presensitization effect in the pig (Murray, Grifo & Parker, 1982) has indicated gains of up to two piglets per litter (increase not significantly significant) in gilts which were presensitized by
insemination with live sperm before puberty, and with killed sperm after puberty. A further preliminary study by Skjervold, Almlid, Onstad & Fossum (1979), in which embryonic survival was higher in sows inseminated with semen to which leucocytes had been added (leucocytes originating from boars of the same breed, boars of a different breed, or from cattle), support the view that immunological factors affect litter size in sows.

An experiment detailed in Chapter 7 further examines the potential to increase reproductive efficiency in sows by uterine presensitization.

2.4.8 Age and parity of the sow

High levels of embryonic and foetal loss occur in breeding sows of all ages, but tend to increase near the fifth litter (Boyd, 1965). This increase compensates for the increase in ovulation rate which continues with increasing parity (Perry, 1954) and results in reduced total numbers of piglets born per litter.

2.4.9 Infection

In Scofield, Clegg & Lamming's (1974) study of embryonic mortality and uterine infection in the pig, flushings of uteri from 46% of all pregnant sows studied were colonized by infection. Bacteria (mainly Escherichia coli and Staphylococcus albus) had previously been isolated from pig uteri by Hajovsky & Gamcick (1966) and by Nikolic (1967). Lancefield Group 'C' streptococci were also isolated by Scofield et al. (1974), although this organism commonly colonizes adult pigs and is associated with a wide range of disease lesions. Similarly, Corynebacterium pyogenes and Pasteurella septica isolated from uteri (Scofield et al., 1974) are usually associated with suppurative lesions such as pneumonia and abscess formation, and establish as secondary infections to other diseases.

As the pathogens present in uterine fluids are unlikely to enter from
the maternal vascular system (Scofield et al., 1974), their presence in the uterus indicates that they enter with boar's preputial fluid, which has a high bacterial content (Reed, 1969), or with semen (Waltz, Foley, Herschler, Tiffany & Liska, 1968). Alternatively, they may be introduced by a penis or insemination catheter which is contaminated with vaginal or faecal flora (Evans, 1967).

While effects of specific pathogens on embryonic tissues are not well understood, subclinical intra-uterine infection and endometritis occurs in a large proportion of sows, and levels of embryonic mortality can be higher in these infected animals (Scofield, 1972; Scofield et al., 1974).

2.4.10 Other factors

Many studies have indicated embryotoxic effects of inhalation anaesthetic gases. While many investigations of these effects have not been adequately controlled, a detailed study by Lane, Nahrwold, Tait, Taylor-Busch, Cohen & Beaudoin (1980) demonstrated foetal reabsorption, skeletal anomalies and macroscopic lesions including encephalocoele, anophthalmia, microphthalmia and gastroschisis in pregnant rats exposed to nitrous oxide on Day 9 of gestation. Xenon, which has anaesthetic properties similar to those of nitrous oxide, did not initiate teratogenic effects when substituted for nitrous oxide in this same study.

Other specific factors have also been associated with growth retardation and death of mammalian embryos, and have been subject to extensive review (e.g. Dawes, 1976; Bustamante, 1980). However, feral pigs, and those domestic pigs which are raised under commercial conditions isolated from the influence of human social diseases, have been spared exposure to anaesthetic gases and other specific embryotoxic elements (e.g. cigarette smoking and alcohol abuse) discussed in these reviews. Further discussion on these factors is thus not appropriate to the objectives of this Thesis.
2.5 HORMONAL STATUS AS A DETERMINANT OF THE OUTCOME OF EARLY PREGNANCY

2.5.1 Introduction

Section 2.4 outlines the more important environmental factors which should be taken into account by managers of early pregnant sows if consistent and high levels of reproductive efficiency are sought; viz.

- Select breeding sows from large litters,
- Mate sows to un-related boars whose off-spring mothered large litters,
- Cull aged sows whose fifth or subsequent litters are small (<8),
- House sows at ambient temperatures between 21 and 28°C during the first 3-4 weeks of pregnancy,
- Condition weaned sows to allow their feed intake to be restricted to about 1.5 kg (21 MJ digestible energy) per day during the first 3 weeks of pregnancy,
- Maintain inbreeding coefficients of sows to below 10%,
- Prior to mating, examine sows for signs of intra-uterine infection and/or endometritis, treat affected sows.
- Avoid anaesthetizing sows during early pregnancy.

Investigations of early pregnancy in sows must take account of these potential determinants of the outcome of early pregnancy, principles which are important to responsible management of pregnant sows. This Thesis applies the principles, but does not further investigate them.

Studies reviewed in the following section indicate that a large proportion of the variance in reproductive efficiency of well-managed sows may be associated with their steroid hormone status during early pregnancy, and that the reproductive efficiency of sows during the first 25 days of gestation can be substantially improved (by about 20%) with appropriate hormone treatment.
2.5.2 Effects of hormone treatment on the survival of pig embryos during early pregnancy

Progesterone has been shown to influence many processes which, collectively, determine how conceptus tissues will develop. For example, progesterone is important in regulating qualitative and quantitative properties of endometrial secretions (Murray et al., 1972; Knight, Bazer & Wallace, 1973; Schlosnagle et al., 1974; Adams, Bazer & Roberts, 1981) which comprise histotroph, the hydrophilic intra-uterine milieu which bathes and nourishes peri-implantation blastocysts during the exponential growth phase between Days 11 and 17 after coitus (Anderson, 1978; Geisert, Brookbank, Roberts & Bazer, 1982; Wright et al., 1983). During this same period, the highest rates of secretion of uterine specific protein by the endometrium of un-mated sows occurs when plasma progesterone concentrations are highest (Murray et al., 1972).

This scenario has provided the basis to proposals that diminished progesterone status may be responsible for a component of preimplantation embryonic loss in pigs. In this regard, Schmidt & Arbeiter (1964) measured progesterone content of CL of sows between Days 18 and 21 after mating and concluded that about 20% of total embryonic loss in pigs is associated with progesterone deficiency, and that this loss occurs between fertilization and implantation. In support of this conclusion, Glasgow, Mayer & Dickerson (1951) and Mayer, Glasgow & Gawienowski (1961) reported a significant positive correlation between the concentration of progesterone excretion products and the levels of embryonic survival in sows. In their investigation of effects of hormone treatment on chorionic outgrowth in sows, McGovern, Morcom, de Sa & Dukelow (1981) observed a 15% increase in chorionic surface area in response to daily injections of 25 mg progesterone and 12.5 ug oestrone, between Days 14 and 23 of gestation. This rise was, however, transient and conferred no detectable benefit to developing
foetuses.

Since Glasgow, Mayer & Dickerson's report (1951), numerous investigators have examined effects of exogenous progestagens and oestrogen administration on embryonic survival, the results of which are summarized in Table 3. These results indicate how different hormone treatment protocols can be associated with widely divergent levels of early embryonic survival. In specific cases cited, hormone treatment reduced levels of early embryonic mortality to less than 10% (Haines, Warnick & Wallace, 1958; Spies et al., 1959), compared to levels of about 25% in control animals (Table 1, Text-figure 1).

Despite this implied potential to improve the efficiency of early pregnancy by modifying the endocrine status of the sow, the roles of hormones in those components of embryo/maternal interaction which affect embryo survival remain poorly understood. The inconsistencies in outcomes of treatments summarized in Table 3 may thus reflect an inability of investigators to identify those sows, or groups of sows, which will respond to hormone treatment. As an extreme example, treatment of early pregnant sows with progesterone can increase (e.g. Schmidt & Arbeiter, 1964) or reduce (e.g. Spies et al., 1959) embryonic survival.

There is no evidence to suggest that steroid hormone treatment 'rescues' genetic variants.

Microscopic examination of uterine flushings collected from sows during the phase of trophoblast proliferation reveals widely varying levels of membrane expansion between blastocysts contained within the same horn (Text-figure 3) and the appearance of 'runt' blastocysts during this developmental period may be consistent with nutritional constraints on trophectoderm growth. Furthermore, this membrane is the active surface for endocytosis of histotroph (Fazleabas, Bazer & Roberts, 1982; Geisert et al., 1982 a), so the effects of retardation of its proliferation will compound in a milieu in which nutrients are limiting. It is thus proposed that 'runt' blastocysts are genetically competent and that their occurrence in
Table 3: Effects of substitution or supplementation of steroid hormones on embryonic survival during the first 25 days of gestation in sows

<table>
<thead>
<tr>
<th>Period (days after mating)</th>
<th>Treatment</th>
<th>Embryonic survival</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Progestagen (umoles/day)</td>
<td>Oestrogen (nmoles/day)</td>
</tr>
<tr>
<td>Sammelwitz, Dziuk &amp; Nalbandov (1956)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>progesterone</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-26</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>&quot;</td>
<td>159</td>
<td>0</td>
</tr>
<tr>
<td>&quot;</td>
<td>318</td>
<td>0</td>
</tr>
<tr>
<td>&quot;</td>
<td>636</td>
<td>0</td>
</tr>
<tr>
<td>&quot;</td>
<td>1272</td>
<td>0</td>
</tr>
<tr>
<td>Haines, Warnick &amp; Wallace (1958)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>progesterone</td>
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<td></td>
</tr>
<tr>
<td>3-25</td>
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<tr>
<td>&quot;</td>
<td>40</td>
<td>0</td>
</tr>
<tr>
<td>Spies, Zimmerman, Self &amp; Casida (1958)</td>
<td></td>
<td></td>
</tr>
<tr>
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..Table 3 continued

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</tr>
<tr>
<td>progesterone</td>
</tr>
<tr>
<td>15-25</td>
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<tr>
<td>OX day 15</td>
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</table>

<table>
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<tr>
<td>progesterone</td>
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<td>15-25</td>
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<tr>
<td>15-25</td>
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<tr>
<td>Day, Romack &amp; Lasley (1963)</td>
</tr>
<tr>
<td>progesterone caproate (Given as single implants)</td>
</tr>
<tr>
<td>E2 benzoate</td>
</tr>
<tr>
<td>11-24</td>
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<tr>
<td>&quot;</td>
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<td>11-24</td>
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<td>7-24</td>
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Text-figure 3: Photomicrograph of blastocysts flushed from the uterus of a gilt 13 days after mating. Note differences in trophoblast proliferation between the two blastocysts.

Magnification approximately 25 x.
blastocyst populations reflects an inability of the mother to provide an intra-uterine environment which will support uniform trophoblast proliferation, embryo migration and successful implantation by all blastocysts. As the rate of embryo attrition is highest during the trophoblast proliferation phase (Scofield et al., 1974; Text-figure 1), and 'runts' become progressively less able to compete for nutrients as their littermates elongate (the surface area, rate of change in surface area, and rate of change in surface area/volume ratio will be diminished in 'runts'), it is suggested that the high rates of blastocyst attrition which occur between Days 10 (hatched and expanded spherical blastocysts) and 15 (filamentous blastocysts; Text-figure 2) of gestation reflects smothering of 'runts' by elongated littermates.

Hormones can influence quantitative and qualitative properties of the intra-uterine milieu (see Chapter 2.3.2), and can enhance embryo survival if administered to pregnant sows during the phase of trophoblastic outgrowth (Table 3). It is now proposed that where hormone treatment has increased embryonic survival in early pregnant sows (particularly treatments during the elongation phase; e.g. Reddy, Mayer & Lasley, 1958; Wildt et al., 1976), the effects are mediated via modification of the intra-uterine milieu to remove nutritional constraints to trophoblast outgrowth, and that hormone treatment could reduce the incidence of 'runts' and raise levels of embryo survival in a large proportion of sows if treatments and indications for treatment were better defined. The same criteria could contra-indicate hormone treatment where subjects are shown to meet a predefined hormone status and, retrospectively, may account for the mosaic of outcomes shown in Table 3.

The final section of this Chapter reviews origins, effects and interactions of steroid hormones during early pregnancy in the sow.
2.5.3 Endogenous sex steroids during early pregnancy of sows

2.5.3.1 Origins

Ovaries

Ovulation is suppressed throughout gestation in the pig (110-118 days, mean 115 days), although some follicular growth and atresia takes place during early pregnancy (Dufour & Fahmy, 1974). Luteal lifespan in the pregnant pig is prolonged, at least in part, by the secretion of a luteotrophic protein hormone complex which is derived principally from the pituitary (Heap, 1972).

The main steroid secreting tissues in ovaries of pregnant sows are the CL, interstitial tissue and the theca interna of unruptured follicles (ibid). The size and steroidogenic activity of CL in the ovaries of pregnant pigs are comparable to these characteristics of CL during the luteal phase of the oestrous cycle (up to 17 days after ovulation; ibid).

The number of CL during pregnancy provides an accurate estimate of the number of ova shed at the oestrus of conception (Perry & Rowlands, 1962; Longenecker, Waite & Day, 1968) and, as a result of early embryonic mortality, usually exceeds the number of embryos.

Progesterone, the most active of the naturally occurring progestagens, is produced by ovaries of pregnant pigs in greater quantities than any other steroid (Heap, 1972) and is derived principally from ovarian luteal tissues (Erb, Nofziger, Stormshak & Johnson, 1962; Nara, Darmadja & First, 1981). The capacity for CL to secrete progesterone is correlated with the rate of luteal blood flow (Niswender, Menon & Jaffe, 1972; Nett, McClellan & Jaffe, 1976). The mass of luteal tissue in pregnant sows is near 8 g, increasing with advancing pregnancy (Dufour & Fahmy, 1974), and with a progesterone content of about 130 nmol/g (Erb et al., 1962). During pregnancy, the total ovarian progesterone content is near 1.0 umol (Dufour & Fahmy, 1974).
Smaller quantities of progesterone are present in the ovarian stroma (about 8 nmol/g) and adrenals (about 13 nmol/g; Erb et al., 1962).

During early pregnancy, peripheral progesterone levels range between 70 nM (on about Day 14 after mating) and 41 nM (on about Day 25 after mating; Guthrie, Henricks & Handlin, 1972; Ekgvist, Einarsson & Settergren, 1974; Robertson & King, 1974; Ash & Heap, 1975; Baldwin & Stabenfeldt, 1975; Parvizi, Elsaesser, Smidt & Ellendorff, 1976).

Active CL are essential to maintain pregnancy in the pig (Short, 1956; du Mesnil du Buisson & Dauzier, 1957; First & Staigmiller, 1973; Nara, Darmadja & First, 1981) and ovariectomy, or any other factor which causes luteal failure and/or cessation of progesterone production, terminates pregnancy within 24-48 h (Ellicot & Dziuk, 1973; First & Staigmiller, 1973; Diehl & Day, 1974; Nara, Darmadja & First, 1981). Progesterone replacement therapy can prevent termination of pregnancy in mated, ovariectomized, sows (Day et al., 1959; Diehl & Day, 1974; First & Staigmiller, 1974; Rice, Ackland & Heap, 1981).

Pituitary

Hypophysectomy causes abortion in early pregnant sows (Kraeling & Davis, 1974), the CL of pregnancy being dependent upon continuous pituitary support. The pituitary luteotrophin is assumed to be luteinizing hormone (LH), as treatment of pregnant sows with anti-LH serum causes luteal regression and termination of pregnancy (Spies, Slyter & Quadri, 1967). Furthermore, LH-containing preparations will maintain CL in hysterectomized/hypophysectomized sows (Anderson & Melampy, 1967).

Basal levels of LH in plasma during pregnancy are near 1-2 ng/ml, higher than the basal level during the oestrous cycle (Guthrie, Henricks & Handlin, 1972; Parvizi et al., 1976). Small peaks of LH are seen at intervals of a few hours during pregnancy and are followed closely by rises in progesterone (see Wrathall, 1980).
Luteal function is regulated largely by the interaction of LH with LH-receptors on luteal cells (Niswender, Menon & Jaffe, 1972; Hansel, Concannon & Lukaszewska, 1973), the concentration of LH to which luteal cells are exposed being important in determining the level of stimulation of progesterone synthesis in porcine CL (Lemon & Loir, 1977). The concentration of luteal LH-receptors is depressed during early pregnancy in the pig (Ziecik, Shaw & Flint, 1980), suggesting down-regulation of luteal-cell function by circulating LH, concentrations of which are elevated in plasma during early pregnancy.

Pituitary prolactin does not appear to affect ovarian endocrine function during early pregnancy in sows (Threlfall, Dale & Martin, 1974; Wrathall, 1980).

Conceptus tissues

Steroidogenic potential. Steroidogenic potential in mammalian blastocysts was first indicated in 1966, with the demonstration, by Huff and Eik-Ness, that 6-day old rabbit blastocysts have the metabolic capacity to convert acetate to pregnenolone, and to metabolize pregnenolone, 17-α-hydroxypregnenolone, progesterone and androstenedione. In 1973, Perry, Heap & Amoroso demonstrated the presence of aromatase activity in pig blastocysts, and later studies indicated a capacity for dispersed trophectoderm tissues from preimplantation pig blastocysts to release oestrone and oestradiol in vitro (Heap, Perry, Gadsby & Burton, 1975; Gadsby, Burton, Heap & Perry, 1976; Perry, Heap, Burton & Gadsby, 1976; Gadsby, Heap & Burton, 1980; Heap, Flint, Hartman, Gadsby, Staples, Ackland & Hamon, 1981). Cultured pig blastocyst tissues can synthesize oestradiol from pregnenolone or progesterone, and synthesize oestrone and oestradiol from dehydroepiandrosterone (DHEA) or androstenedione (Gadsby et al., 1976). Further studies (e.g. Gadsby, 1978) have shown that neutral steroids are rapidly metabolized to other neutral compounds by dispersed blastocyst
tissues, and that certain enzymes involved in the steroidogenic cascade (e.g. 3- $\beta$-HSD) are active in pig embryonic tissues from 8-cell morula stages (Dickmann, Dey & Sen-Gupta, 1976) through to filamentous stages (Flood, 1974).

With the exception of the side-chain cleavage enzymes responsible for the conversion of cholesterol to pregnenolone, the full complement of enzymes necessary for the synthesis of oestrogens from acetate is present in pre-implantation trophoderm tissues of pig blastocysts (Heap, Flint, Gadsby & Rice, 1979). The presence of unconjugated oestrogens in pig blastocyst tissues (Perry, Heap & Amoroso, 1973; Dickmann, 1975; Gadsby & Heap, 1978) supports the evidence for oestrogen synthesis by pig blastocysts in vivo. Progesterone, also, has been identified in blastocyst tissues of the pig (Gadsby & Heap, 1978) and of other species (Seamark & Lutwak-Mann, 1972).

Time of onset of steroid synthesis by pig blastocysts. Neither DHEA nor androstenedione can be incorporated into oestrone or oestradiol by cultured tissues of Day 10 pig blastocysts (about 2 mm diameter; Heap et al., 1975). However, incubations of dispersed tissues of blastocysts collected from sows 12 days after mating (about 5 mm diameter) show substantial incorporation of DHEA into oestrone and oestradiol. This incorporation is maintained at a relatively constant rate until after Day 20 of gestation (ibid).

The induction of aromatase, detectable in vitro on Day 12 after mating (Heap et al., 1975), seems to precede blastocyst elongation. That is, oestrogen synthesis has been detected in spherical blastocysts, and oestrone and oestradiol can be detected in spherical and ovoid blastocysts (Heap et al., 1979; Heap et al., 1981). Aromatase activity in intact blastocysts is only detectable after elongation has commenced (Flint, Burton, Gadsby, Saunders & Heap, 1979).

High concentrations of progesterone have been measured in blastocyst tissues at about Day 12 of gestation, arising either from synthesis by
trophectoderm cells (Gadsby & Heap, 1978), or from uptake of progesterone from the intrauterine environment. Progesterone can be utilized by embryonic tissue as a substrate to oestrogen synthesis during early pregnancy, radio-labelled oestrone being detectable in foetal membranes after infusion of $^{3}H$-progesterone into the uterine artery on Day 22 of pregnancy (Flint et al., 1979).

While embryonic tissues of the pig acquire the enzymic machinery to convert neutral steroids into oestrogens at about Day 12, this capacity appears to be modified as pregnancy progresses (Heap et al., 1979). For example, the conversion of $C_{19}$ steroids to oestrogens declines per unit weight of embryonic tissue as pregnancy progresses (Perry et al., 1976), reflected in sharp changes in levels of oestrogens in maternal plasma during pregnancy (Robertson & King, 1974). During late gestation, neutral $C_{19}$ steroids are converted into oestrogens by placental tissues only (Ainsworth & Ryan, 1966).

Placenta

The placenta of the pregnant pig can synthesize a series of steroid hormones, the most significant of which are the oestrogens (Heap, 1972). DHEA sulphate, originating from the foetal adrenal, appears to be the most likely precursor to placental oestrogen synthesis (Thau & Lanman, 1975).

Endometrium

Sulphotransferase (oestrone-3-sulphotransferase) activity in the endometrial tissues of pregnant sows increase with rising plasma progesterone levels, and is maintained at high levels for about the first three weeks of gestation (Pack, Brooks, Dukelow & Brooks, 1979; Dwyer & Robertson, 1980). This activity leads to endometrial sulphoconjugation of oestrogens of placental and conceptus origin (Robertson, King & Dyck, 1978).
2.5.3.2 Release of steroids synthesized by the embryo/maternal complex

Oestrogens synthesized in the placenta, or by blastocyst tissues, are sulphoconjugated in the endometrium (Pack & Brooks, 1974; Pack et al., 1979; Dwyer & Robertson, 1980; Meyers, Lozon, Corombos, Saunders, Hunter, Christensen & Brooks, 1983) and released to the uterine venous drainage as sulphoconjugates (Robertson & King, 1974). Peripheral plasma levels of oestrogens (particularly oestrone sulphate) in early pregnant sows exceed non-pregnancy values before Day 17 after mating (Robertson, King & Dyck, 1978; Stone & Seaman, 1985) and rise to maximum values between Days 25 and 30 of pregnancy (Robertson & King, 1974), coinciding with a peak in urinary oestrogen levels (Lunaas, 1962; Raeside, 1963).

The foeto-placental unit is the principal source of plasma oestrogens during early pregnancy and during the third trimester of pregnancy in sows (Ainsworth, 1972; Challis & Thorburn, 1975).

Prior to their renal excretion, unconjugated plasma oestrogens are conjugated to glucuronides and, to a lesser extent, to sulphates in the maternal liver (Thau & Lannan, 1975).

2.5.3.3 Endocrine aspects of the maternal recognition of pregnancy

The signal which leads to luteal maintenance in the pregnant sow is transferred from conceptus to maternal tissues between Days 10 and 12 of gestation (Dhindsa & Dziuk, 1968). When blastocysts are removed from one uterine horn on Day 12 after mating, or later, pregnancy is established in the contralateral horn, ligated to prevent transuterine migration. Pregnancy is not established if conceptus tissue is completely flushed from one uterine horn before Day 12 (ibid), although unilateral gestation can be prolonged by daily injection of progesterone (about 625 umoles/day; du Mesnil du Buisson, 1961).
The timing of maternal recognition of pregnancy coincides with elongation of blastocysts (Anderson, 1978) and the appearance of aromatase activity in these tissues (Perry et al., 1976; Flint et al., 1979). Accumulation of oestrone and oestradiol in spherical blastocysts (Gadsby & Heap, 1978) precedes demonstrable aromatase activity in blastocyst tissues, and precedes the divergence in concentrations of oestradiol in utero-ovarian (UOV) venous plasma between pregnant and non-pregnant sows (Moeljono, Thatcher, Bazer, Frank, Owens & Wilcox, 1977). Elevated concentrations of oestrogens in UOV venous plasma of oestrogen-treated, non-pregnant, sows are associated with reduced concentrations of PGF2-α in UOV venous plasma, to levels similar to those measured between Days 12 and 20 of pregnancy (Frank, Bazer, Thatcher & Wilcox, 1977; Moeljono et al., 1977). In view of the established luteolytic effects of PGF2-α in the pig after Day 12 of pregnancy (Gleeson, 1974; Guthrie & Polge, 1976), the effect of peripheral oestrogen in reducing circulating PGF2-α levels is consistent with a role for blastocyst-derived oestrogens in extending luteal lifespan in pregnant animals. In regard to this effect, Frank et al. (1977) have proposed that oestrogens affect PG-secretory mechanisms within the uterus such that PGF2-α is accumulated in the uterine lumen rather than released to the uterine venous drainage. The presence of aryl sulphatase activity in blastocyst tissues could ensure that oestrogens secreted by blastocysts are unconjugated and therefore active as anti-luteolysins within the endometrial tissues (Perry et al., 1976; Heap et al., 1979). This activity is then attenuated in peripheral tissues through sulphonyl conjugation of oestrone in the endometrium (Pack & Brooks, 1974), prior to release to uterine venules.

Initial stages of the maternal recognition of pregnancy appear to depend upon local anti-luteolytic effects of oestrogens derived from the embryo/placental complex, and the CL of pregnancy require luteotrophic support after Day 14 of pregnancy, even in the absence of the uterine luteolysin (du Mesnil du Buisson & Denamur, 1968). Oestrogens, or other conceptus-derived substances, may thus affect luteal activity through a
systemic route and, in this respect, oestradiol benzoate can protect the CL against luteolytic effects of exogenously administered PGF2-α in hysterectomized sows (Kraeling, Rampacek & Ball, 1975). Furthermore, oestrogens (particularly sulphoconjugated oestrone) are potent luteotrophins in the sow (Gardner, First & Casida, 1963; Bazer & Thatcher, 1977; Frank, Bazer, Thatcher & Wilcox, 1978; Ford & Magness, 1980) and stimulate uterine arterial blood-flow (Dickson, Bosc & Locatelli, 1969; Ford, Christenson & Ford, 1982), both effects being consistent with maternal recognition and maintenance of pregnancy.

Injection of pregnant sows (between Days 10 and 21 post coitum) with antisera to oestrone and oestradiol does not impair CL maintenance or implantation, providing circumstantial evidence that unconjugated oestrogens in the peripheral circulation are not luteotrophic (Robertson, Dwyer & King, 1980). The antisera used in this study did not cross-react with oestrogen sulphoconjugates.

The maternal recognition of pregnancy in the pig is thus proposed to involve an initial suppression of PGF2-α secretion into the uterine venous drainage, at a time when luteal sensitivity to this luteolysin is increasing. Subsequently, a rising production of oestrogens by the preimplantation embryo may exert a luteotrophic influence, possibly augmented through maintenance of luteal LH receptors (Flint et al., 1979), mediating the luteotrophic action of LH secreted by the maternal pituitary.
2.6 Discussion

Pig blastocyst tissues can synthesize steroids which are essential to the recognition of pregnancy (Chapter 2.5.3). Steroids of blastocyst origin also provide luteotrophic support and appear to trigger a mechanism which blocks luteolysis, both functions being essential to effective maintenance of pregnancy in this species (Chapter 2.5.3). Furthermore, steroid hormones can influence quantitative and qualitative properties of the intrauterine milieu and can reduce embryonic survival if administered to sows during early pregnancy (Chapter 2.5.2). The capacity for conceptuses to release steroid hormones may thus be essential to normal growth and development, and current evidence suggests that this capacity may be related to the likelihood of preimplantation embryos attaining their genetic growth potential.

Manipulation of endocrine components of the embryo/maternal complex to stimulate steroidogenesis by ovarian, endometrial and/or blastocyst tissues, or to supplement steroid release by blastocysts, should thus advantage those blastocysts whose growth is constrained by nutrient pools (Chapter 2.3) or any other steroid-dependent embryotrophic component of the embryo/maternal complex (Chapter 2.5.2). This, in turn, should reduce the incidence of blastocyst 'runts' (Text-figure 3) and increase the proportion of conceptuses which survive the critical pre-implantation development phase between Days 9 and 15 of pregnancy (Text-figure 1).

Experiments detailed in this Thesis aim to elucidate components of the embryo/maternal complex, to provide practical bases for treatment of sows to increase the proportion of fertilized embryos which can successfully implant.
EXPERIMENTAL
EXPERIMENTAL

CHAPTER III

POTENTIAL DETERMINANTS OF EMBRYONIC GROWTH DURING THE
PRE-IMPLANTATION PERIOD

3.1 INTRODUCTION

Blastocysts which fail to elongate prior to implantation are at a competitive disadvantage prior to and at implantation, and it is now proposed (Chapter 2.5.2) that this retardation could account for a proportion of the high level of embryonic loss which occurs between Days 9 and 15 of pregnancy in sows (about 30%; Scofield et al., 1974; see Text-figure 1).

As growth factors for protein anabolism in pre-implantation blastocysts in vivo are provided from endometrial secretions via the uterine 'milk', the experiments described in this Chapter attempt to identify specific determinants of pre-implantation blastocyst growth by describing levels of steroid hormones and of free amino acids in uterine flushings collected from gilts during early pregnancy. Levels of these elements in histotroph collected during the pre-implantation phase of pregnancy should then reflect peculiarities in substrate requirements for elongation of porcine blastocysts in vivo.
3.2 STEROID HORMONES IN HISTOTROPH

3.2.1 Introduction

Effects of androgens (particularly testosterone) as protein anabolic agents are well established. Furthermore, steroids can influence quantitative and qualitative properties of endometrial secretions (predominantly progesterone and oestrogens; Schlosnagle et al., 1974; Basha, Bazer & Roberts, 1979; Aitken, 1979; Adams, Bazer & Roberts, 1981; Fazleabas, Bazer & Roberts, 1982; Geisert et al., 1982 a; see Chapter 2.3) and may thus be important in maintaining pools of endometrial proteins and free amino acids in histotroph, for assimilation into conceptus proteins.

Conceptus tissues of pigs have the capacity to synthesize steroids (see 2.5.3.1), yet precursors to steroid synthesis by pre-implantation embryos in vivo have not been defined. In this regard, and in respect to evidence that endometrial secretion is stimulated during pregnancy in the pig (Knight, Bazer & Wallace, 1973; Basha, Bazer & Roberts, 1980; Geisert et al., 1982 a), the experiment below determines levels of steroids in washings of uteri of pregnant sows, and compares these values with respective steroid levels in uterine fluids collected from un-mated gilts between Days 9 and 15 after oestrus, prior to the normal onset of luteolysis (Henricks, Guthrie & Handlin, 1972). Further comparison is made to the steroid content of plasma.

3.2.2 Materials and methods

3.2.2.1 Animals

Thirty-two Large White sows (16 un-mated and 16 mated) were slaughtered 9 to 15 days after the day of onset of the previous oestrus. Immediately after recovery of uteri, each uterine horn was cannulated above the bifurcation
and flushed from the utero-tubal junction, towards the cannula (Stone, Whyte, Pointon, Quinn & Heap, 1984), with 10 ml 0.9% (w/v) NaCl at 4°C. Flushings were transported in ice to the laboratory and there centrifuged at low speed (56 g, 20 min, 4°C) to precipitate conceptus tissue and/or other particulate matter. Supernatants were portioned and stored at -15°C. Precipitates from flushings of tracts of mated gilts were examined microscopically to confirm the presence of conceptus tissues consistent with stage of pregnancy (Anderson, 1978). Numbers of CL associated with individual uterine horns were recorded.

Blood samples (mixed arterial and venous) collected from all animals at exsanguination were centrifuged (2011 g, 20 min, 4°C) and plasma samples were stored at -15°C.

Concentrations of steroid hormones in samples of plasma and in flushings from each uterine horn were determined by radioimmunoassay, according to the methods detailed below.

Days of slaughter were organized to provide post-mortem samples from a minimum of two gilts on each of Days 9 through 15 after oestrus/coitus.

3.2.2.2 Assay methods

General

Solvents for extraction were of analytical reagent grade and were redistilled before use. The solvent:sample ratio (v/v) exceeded 10:1 in all assays. Solvent was removed from sample extracts at 37°C under air.

Unconjugated steroid standards were purchased from Steraloids (Wilton, NH, U.S.A.) and pregnenolone sulphate (sodium salt) and oestrone-3-sulphate (potassium salt) were obtained from Sigma Chemical Company (St Louis, MO, U.S.A.). Standard steroids were not recrystallized further.

In assays for pregnenolone sulphate, DHEA, DHEA sulphate and oestrone sulphate in plasma and in uterine washings, evaporated standards were
equilibrated with steroid-free plasma or saline respectively (volumes equivalent to sample volume), then extracted and assayed with the uterine washing/plasma samples.

Before assay of pregnenolone, progesterone, androstenedione, oestrone, testosterone and oestradiol, solvent extracts of plasma and uterine flushings were fractionated by column chromatography (hydroxyalkoxypropyl Sephadex; Lipidex, Packard Instrument Company, IL, U.S.A.; column bed dimensions 80 mm x 3 mm), as detailed below.

For pregnenolone and progesterone, samples (250 ul) were extracted with 2.5 ml hexane:ethyl acetate (9:1 v/v). The solvent was removed and residues redissolved in 200 ul hexane:ethyl acetate (9:1 v/v) and carefully loaded onto the column bed. Elution was made with 1.0 ml hexane (discard), 2.5 ml hexane (progesterone fraction, 77% recovery), a further 1.0 ml hexane (discard) and 2.5 ml hexane:ethyl acetate (9:1 v/v; pregnenolone fraction, 93% recovery).

For androstenedione, testosterone, oestrone and oestradiol, samples (400 ul) were extracted with 2.5 ml heptane:ethyl acetate (2:3 v/v), the solvent removed and residues redissolved in 200 ul hexane:ethyl acetate (9:1 v/v) and loaded. Elution was made with 0.5 ml hexane:ethyl acetate (9:1 v/v; discard), 2.0 ml hexane:ethyl acetate (9:1 v/v; androstenedione fraction, 71% recovery), 2.0 ml hexane:ethyl acetate (9:1 v/v; testosterone fraction, 93% recovery), 2.5 ml hexane:ethyl acetate (4:1 v/v; oestrone fraction, 84% recovery) and 2.0 ml ethyl acetate (oestradiol fraction, 96% recovery).

Tritiated tracers were purchased from New England Nuclear (Boston, MA, U.S.A.).

The assay buffer contained 6.1 g NaH₂PO₄·2H₂O, 10.9 g Na₂HPO₄·2H₂O, 9.0 g NaCl, 1.0 g NaN₃ and 1.0 g bovine serum albumin (BSA, Sigma) per litre, and was pH adjusted to 7.0.

After incubation of sample/standard extracts with tracer and antiserum, antibody-bound steroid was precipitated by the addition of 1 mg gamma-globulin and polyethylene glycol 6000 (to provide a final
concentration of 20.6%). After vortexing, tubes were centrifuged (4°C, 3574 g, 20 min) and the supernatant, containing free steroid, was aspirated and discarded.

Precipitated pellets, containing protein-bound steroid, were resuspended in 1.8 ml Scintisol (Isolab Incorporated, Akron, OH, U.S.A.), and radioactivity was measured in a Tracer Analytic 6891 liquid scintillation spectrometer.

Intra-assay coefficients of variation were <10% in all assays. All uterine washing and plasma samples were assayed in single assays with blanks derived from saline or steroid-free pig plasma respectively.

Specific assays

Pregnenolone. After chromatography, solvent was removed from the pregnenolone fraction and pregnenolone was determined using an antiserum raised in goats against pregnenolone-3-carboxymethyloxime (CMO)-gamma globulin. The cross-reactivity of the antiserum, used at a final dilution of 1:16000, was 37.5% with progesterone, 9.95% with 5α-pregnan-3-ol-20-one, 4.44% with 5α-pregnan-3,20-dione, 0.21% with 17α-hydroxyprogesterone, 0.18% with DHEA, 0.15% with DHEA sulphate, and <0.10% with 17α-hydroxypregnenolone, 20α-hydroxypreg-4-ene-3-one, androstenedione, oestradiol, oestrone, oestrone sulphate and testosterone. The limit of sensitivity of the assay was 0.1 pmol per tube.

Pregnenolone sulphate. Unconjugated steroids were extracted from histotroph/plasma samples with 2.0 ml hexane:ethyl acetate (9:1 v/v) and discarded. The remaining pellet was saturated with NaCl and extracted with 2.5 ml ethyl acetate saturated with distilled water, the solvent was removed and the residue assayed using the same antiserum as employed in pregnenolone immunoassay. Levels of unconjugated steroids present in the pregnenolone sulphate-containing extracts were <5% of levels in the original media. For
assay, extracts were dispensed into polypropylene tubes, into which any unconjugated steroid would be adsorbed (Bruning, Jonker & Boerema-Baan, 1981), further diminishing any contribution of these compounds to levels of pregnenolone sulphate determined by this radioimmunoassay technique. The limit of sensitivity of the assay was 0.2 pmol pregnenolone sulphate per tube.

DHEA. Samples were extracted with 2.5 ml heptane:ethyl acetate (2:3 v/v), the solvent was removed and DHEA determined using an antiserum raised in goats against DHEA-15β-(3-thioproprionic acid)-BSA. The cross-reactivity of the antiserum, used at a final dilution of 1:5000, was 0.20% with androstenedione, 0.09% with DHEA sulphate, 0.06% with testosterone and <0.04% with 5α-dihydrotestosterone, progesterone, oestradiol and pregnenolone. The limit of sensitivity of the assay was 0.1 pmol per tube.

DHEA sulphate. Unconjugated steroids were extracted from samples with heptane:ethyl acetate (2:3 v/v) and discarded. The remaining pellet was saturated with NaCl and extracted with 2.5 ml ethyl acetate saturated with water. Dried extracts were assayed for DHEA sulphate using an antiserum raised in goats against DHEA-hemisuccinate-BSA. The cross-reactivity of the antiserum, used at a final dilution of 1:1000, was 67.3% with DHEA, 10% with androstenedione, 0.08% with testosterone, 0.07% with dihydrotestosterone and <0.02% with progesterone and oestradiol. Levels of these unconjugated steroids in the DHEA sulphate-containing extracts were <2% of levels in the original media. The limit of sensitivity of the assay was 0.1 pmol per tube.

Progesterone. After removal of solvent from the chromatography fractions, residues were assayed for progesterone using an antiserum raised in goats against progesterone-11α-hemisuccinate-gamma globulin. Used at a final dilution of 1:7000, the cross-reactivity of the antiserum was 1.2% with
17α-hydroxyprogesterone, 0.44% with pregnenolone, 0.17% with 20α-hydroxyprog-4-ene-3-one and <0.07% with androstenedione, cortisol, testosterone and oestradiol. The limit of sensitivity of the assay was 0.1 pmol per tube.

**Androstenedione.** Solvent was removed from the chromatography fractions and residues were assayed for androstenedione using an antiserum raised in sheep against androstenedione-3-CMO-BSA. Used at a final dilution of 1:75000, the cross-reactivity of the antiserum was 2.6% with DHEA, 1.18% with testosterone, 0.33% with 5α-dihydrotestosterone, 0.27% with progesterone and 17α-hydroxyprogesterone and <0.03% with oestradiol and cortisol. The limit of sensitivity of the assay was 0.1 pmol androstenedione per tube.

**Oestrone.** Dried chromatography fractions containing oestrone were assayed for oestrone using an antiserum raised in sheep against oestrone-3-CMO-gelatin. Used at a final dilution of 1:30000, the cross-reactivity of the antiserum was 83% with oestrone sulphate (not extracted into the organic phase), 4.2% with oestrone-3-glucosiduronate and <0.01% with 21 other C₁₉ and C₂₁ steroids/conjugates tested. The limit of sensitivity of the assay was 0.05 pmol oestrone per tube.

**Oestrone sulphate.** After extraction of unconjugated steroids with ether, samples were saturated with NaCl, oestrone sulphate was extracted with 2.0 ml tetrahydrofuran and the solvent was removed. Residues were assayed for oestrone sulphate using the same antiserum as was used for oestrone, but at a final dilution of 1:33000. The limit of sensitivity of the assay was 0.01 pmol oestrone sulphate per tube.

**Oestradiol.** After chromatography, solvent was removed from oestradiol fractions and residues were assayed for oestradiol using an antiserum raised in goats against oestradiol-6-CMO-gamma globulin. Used at a final dilution
of 1:30000, the cross-reactivity of this antiserum was 16.9% with oestrone, 1.1% with oestriol and <0.02% with testosterone, androstenedione and progesterone. The limit of sensitivity of the assay was 0.04 pmol oestradiol per tube.

**Testosterone.** Dried chromatography fractions containing testosterone were assayed for testosterone using an antiserum raised in goats against testosterone-15β-(3-thioproprionic acid)-BSA. Used at a final dilution of 1:10000, this antiserum cross-reacted 11.9% with 5α-dihydrotestosterone, 0.3% with androstenedione, 0.24% with progesterone, 0.05% with pregnenolone and <0.02% with DHEA and oestradiol. The limit of sensitivity of the assay was 0.1 pmol testosterone per tube.

**Statistics**

For each steroid measured, statistical differences between the values for pregnant and non-pregnant animals, and between plasma and uterine fluid, were analysed by Student's t test (Clarke, 1969). For individual animals, relationships between numbers of CL and steroid properties of uterine washings (total steroid content and steroid concentration in free uterine fluid) and of plasma were examined by least squares linear regression analysis (Li, 1969). Relationships between these same steroid values and days post coitum and days after oestrus were analysed similarly.

**3.2.3 Results**

Despite efforts to avoid rupture of the fragile trophectoderm tissues during collection of uterine flushings, disrupted membranes were seen when precipitated conceptus tissues were examined microscopically. Uterine washings collected from pregnant gilts in this study therefore include blastocoele fluids and, while the volume of fluid contributed from this
source would be low, concentrations of steroids in blastocoele fluid may be high (Seamark & Lutwak-Mann, 1972; Borland, Erickson & Ducibella, 1977; Gadsby & Heap, 1978) and at variance to concentrations in the uterine fluid which bathes blastocysts in vivo. Regardless, all steroidal components of the fluid intrauterine milieu (or precursors to these steroids) will have originated from maternal pools and differences between steroidal properties of uterine fluids collected from pregnant and from unmated gilts can be attributed to the presence of conceptus tissues. Analysis of these differences is the primary objective of this study. The admixture of histotroph with blastocoele fluid is hereafter referred to as free uterine fluid.

The total recovered flush volume exceeded 10 ml in 24/32 non-pregnant and in 20/32 pregnant uterine horns. Despite earlier evidence that endometrial secretion is enhanced during early pregnancy in sows (Murray et al., 1972; Knight, Bazer & Wallace, 1973; Bazer, 1975; Zavy et al., 1980; Basha, Bazer & Roberts, 1980; Geisert et al., 1982 a), the overall average volume of free uterine fluid (ml; ± s.e.m.) recovered from the gravid uterine horns (11.7 ± 4.3) was not significantly higher than the respective non-pregnant value (10.1 ± 3.6; t test).

Total levels of pregnenolone, progesterone, DHEA, testosterone, oestrone and oestradiol were higher (P<0.05) in washings of pregnant uteri than of non-pregnant uteri between Days 9 and 15 after oestrus (Table 4), reflecting rising values for pregnant gilts after Day 10 post coitum (Text-figure 4). Similarly, significant (P<0.05) differences between pregnant and non-pregnant animals in regard to concentrations of pregnenolone, DHEA, DHEA sulphate, androstenedione, oestrone, oestrone sulphate and oestradiol in free uterine fluids (total volume of intrauterine fluid recovered, minus the volume of saline flush; Tables 5 and 6) can be attributed to divergence of pregnant and non-pregnant values after Day 9 post oestrus (Text-figure 5).

Between Days 9 and 15 after oestrus, average levels of DHEA, DHEA sulphate, androstenedione and oestrone sulphate in plasma of pregnant gilts
Table 4. Total content of steroids in washes of single uterine horns of mated (pregnant, N=32) and of unmated (non-pregnant, N=32) gilts between Days 9 and 15 after oestrus

<table>
<thead>
<tr>
<th>Steroid</th>
<th>Non-pregnant</th>
<th>Pregnant (+)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>C₂₁</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pregnenolone</td>
<td>26.9 ± 6.5</td>
<td>202.1 ± 46.6</td>
</tr>
<tr>
<td>Pregnenolone sulphate</td>
<td>65017 ± 25583</td>
<td>106865 ± 41874</td>
</tr>
<tr>
<td>Progesterone</td>
<td>86.8 ± 27.6</td>
<td>337.0 ± 108.6</td>
</tr>
<tr>
<td><strong>C₁₉</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DHEA</td>
<td>3.70 ± 1.44</td>
<td>18.7 ± 2.1</td>
</tr>
<tr>
<td>DHEA sulphate</td>
<td>2959 ± 1290</td>
<td>937 ± 469</td>
</tr>
<tr>
<td>Androstenedione</td>
<td>14.1 ± 5.1</td>
<td>20.4 ± 6.6</td>
</tr>
<tr>
<td>Testosterone</td>
<td>2.10 ± 1.08</td>
<td>9.40 ± 3.34</td>
</tr>
<tr>
<td><strong>C₁₈</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oestrone</td>
<td>2.92 ± 0.72</td>
<td>13.92 ± 4.05</td>
</tr>
<tr>
<td>Oestrone sulphate</td>
<td>1234 ± 453</td>
<td>404 ± 199</td>
</tr>
<tr>
<td>Oestradiol</td>
<td>0.69 ± 0.21</td>
<td>62.96 ± 32.44</td>
</tr>
</tbody>
</table>

+ Significantly different from value for non-pregnant sows;
* P<0.05; ** P<0.01; *** P<0.001 (Student's t test)
Text-figure 4: Average (± s.e.m.) total levels of steroids in washings of single uterine horns of unmated (○) and mated (●) gilts on individual days between Days 9 and 15 after oestrus. For each day, non-pregnancy and pregnancy values were compared using a t-test.
Table 5. Concentrations of steroids in free uterine fluid and in plasma of un-mated and of mated gilts between Days 9 and 15 after oestrus

<table>
<thead>
<tr>
<th>Steroid</th>
<th>Un-mated gilts</th>
<th>Mated gilts</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Uterine fluid (N=24)</td>
<td>Plasma (N=16)</td>
</tr>
<tr>
<td>Pregnenolone</td>
<td>8.04 ± 1.75</td>
<td>3.36 ± 0.99 *</td>
</tr>
<tr>
<td>Pregnenolone sulphate</td>
<td>1973 ± 533</td>
<td>37.3 ± 8.4 ***</td>
</tr>
<tr>
<td>Progesterone</td>
<td>14.6 ± 5.8</td>
<td>72.7 ± 12.0 ***</td>
</tr>
<tr>
<td>DHEA</td>
<td>0.24 ± 0.06</td>
<td>0.10 ± 0.02 *</td>
</tr>
<tr>
<td>DHEA sulphate</td>
<td>95.2 ± 26.1</td>
<td>0.61 ± 0.10 ***</td>
</tr>
<tr>
<td>Androstenedione</td>
<td>1.26 ± 0.09</td>
<td>0.45 ± 0.04 ***</td>
</tr>
<tr>
<td>Testosterone</td>
<td>0.39 ± 0.12</td>
<td>0.85 ± 0.18 *</td>
</tr>
<tr>
<td>Oestrone</td>
<td>0.92 ± 0.26</td>
<td>0.05 ± 0.01 **</td>
</tr>
<tr>
<td>Oestrone sulphate</td>
<td>47.3 ± 12.1</td>
<td>0.32 ± 0.04 ***</td>
</tr>
<tr>
<td>Oestradiol</td>
<td>0.26 ± 0.12</td>
<td>0.03 ± 0.01</td>
</tr>
</tbody>
</table>

+ : Significantly different from value for free uterine fluid:
* P<0.05; ** P<0.01, *** P<0.001, (Student's t test)
Table 6. Analysis of differences between pregnant and non-pregnant gilts in respect to concentrations of steroids in free uterine fluid and in plasma

<table>
<thead>
<tr>
<th>Steroid</th>
<th>Significance of difference between concentrations of pregnant and non-pregnant gilts (+)</th>
<th>Pregnant/Non-pregnant ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Uterine fluid</td>
<td>Plasma</td>
</tr>
<tr>
<td>C21 Pregnenolone</td>
<td>*</td>
<td>NS</td>
</tr>
<tr>
<td>C21 Pregnenolone sulphate</td>
<td>NS</td>
<td>*</td>
</tr>
<tr>
<td>C21 Progesterone</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>C19 DHEA sulphate</td>
<td>**</td>
<td>**</td>
</tr>
<tr>
<td>C19 Androstenedione</td>
<td>**</td>
<td>**</td>
</tr>
<tr>
<td>C19 Testosterone</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>C19 **</td>
<td>***</td>
<td>NS</td>
</tr>
<tr>
<td>C18 Oestrone</td>
<td>**</td>
<td>NS</td>
</tr>
<tr>
<td>C18 Oestrone sulphate</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>C18 Oestradiol</td>
<td>***</td>
<td>NS</td>
</tr>
</tbody>
</table>

*; NS, not significant, * P<0.05; ** P<0.01; *** P<0.001 (Student's t test)
Text-figure 5: Average (± s.e.m.) concentrations of steroids in free uterine fluid recovered from single uterine horns of unmated (○) and mated (●) gilts on individual days between Days 9 and 15 after oestrus. The data represent only those uterine horns from which free uterine fluid could be recovered (non-pregnant, N=24; pregnant, N=20). For each day, non-pregnancy and pregnancy values were compared using a t-test.
exceeded non-pregnant concentrations while pregnenolone sulphate levels were diminished (P<0.05; Tables 5 and 6). Daily plasma steroid levels between Days 9 and 15 after oestrus/coitus are summarized in Text-figure 6.

Linear regressions between steroid properties of uterine fluids and plasma, numbers of CL, days after oestrus and days post-coitum, were not statistically significant (P>0.10).

3.2.4 Discussion

Significant amounts of steroid hormones were present in uterine washings recovered from un-mated sows between Days 9 and 15 after oestrus (Table 4), with evidence for a marked (50 to 150-fold) concentration of the sulphon conjugates of pregnenolone, DHEA and oestrone in free uterine fluid compared with plasma levels (Table 5). Further accumulation of pregnenolone, progesterone, DHEA, testosterone, oestrone and oestradiol occurred in uterine fluid during pregnancy (P<0.05; Table 4; Text-figure 4).

The higher levels of oestrone and oestradiol in uterine fluids of gilts between Days 9 and 15 of pregnancy (Table 4; Text-figure 4) are consistent with oestrogen synthesis by dispersed pig blastocyst tissues (Heap et al., 1981) and the results concur with those of Ford, Christenson & Ford (1982) in respect to the predominance of oestradiol in uterine fluid (Tables 4 and 5) and their evidence for concentration of oestradiol in uterine venous plasma. Levels of oestrone and oestradiol in the washings of gravid uteri increased after about Day 11 of pregnancy, and were significantly higher (P<0.05) than non-pregnancy values by Day 15 after oestrus (Text-figure 4). This result is in accord with previous reports that oestrogen synthesis by pig blastocyst tissues in vivo is initiated between Days 10 and 12 of gestation (Heap et al., 1975).

Oestrone sulphate levels in uterine fluid were similar for pregnant and non-pregnant animals (Table 4). However, concentrations of oestrone
Text-figure 6: Average (± s.e.m.) concentrations of steroids in plasma of unmated (○) and mated (●) gilts on individual days between Days 9 and 15 after oestrus. For each day, non-pregnancy and pregnancy values were compared using a t-test.
sulphate in plasma were elevated in pregnancy ($P<0.05$; Table 5; see Text-figure 6), this being consistent with oestrone secretion by blastocyst tissues as proposed by Heap et al. (1981), its accumulation in histotroph ($P<0.001$; Table 5; Text-figure 4), sulphation in uterine or other maternal tissues (Pack & Brooks, 1974; Pack et al., 1979; Meyers et al., 1983), and release to the maternal circulation as the sulphonyl conjugate.

The preferred precursor and pathway for oestrogen synthesis by pig conceptuses have not previously been identified. However, since ovarian progesterone is not an obligatory precursor, Flint et al. (1979) and Heap et al. (1981) have proposed that oestrogens are synthesized by the blastocyst from adrenal progesterone or pregnenolone, or from cholesterol or acetate. The immediate oestrogen precursor measured in the present study, androstenedione, was concentrated in uterine fluid relative to plasma in pregnant and in non-pregnant gilts ($P<0.01$; Table 5), although mechanisms inducing the pregnancy-associated increases in concentration of this steroid in plasma and in uterine fluid ($P<0.001$ and $P<0.01$ respectively; Tables 5 and 6; see Text-figures 5 and 6) remain to be determined. Furthermore, the accumulation and concentration of androgens in uterine fluid between Days 9 and 15 of pregnancy (Tables 4, 5 and 6; Text-figures 4 and 5) provide a potentially important source of oestrogen precursor, but whether these events reflect changes in enzyme activity in maternal or embryonic tissues remains unresolved. Besides being potential oestrogen precursors, androstenedione and testosterone may be important in their own right, as both are potent protein anabolic agents which may play a role in the exponential increase in protein content of pig embryos between Days 9 and 15 after coitus (Anderson, 1978; Wright et al., 1983).

Pig blastocyst tissues show aryl sulphotase activity (Flint et al., 1979) and are able to synthesize oestrogen from pregnenolone and DHEA in vitro (Heap et al., 1981). The steroid sulphonyl conjugates are also, therefore, potential precursors for blastocyst steroidogenesis and, with the high concentration of pregnenolone sulphate (3.81 μM) and of DHEA sulphate
(13.3 nM) in free uterine fluid of pregnant gilts (Table 5), oestrogen synthesis from these sources must be considered.

The accumulation and concentration of the sulphoconjugates of pregnenolone and of DHEA in uterine fluid of un-mated animals (P<0.001; Tables 4 and 5) indicates a concentrating mechanism for sulphoconjugates which is specific to maternal tissues. Further accumulation of pregnenolone sulphate in uterine fluid during pregnancy (Tables 4 and 6; Text-figure 4) could then be attributable to induction of further sulphotransferase activity during early pregnancy (Dwyer & Robertson, 1980) by progestagens or other pregnancy-associated hormones, as previously demonstrated in cultured human endometrium (Clarke, Adams & Wren, 1982). As average pregnenolone sulphate concentrations were lower in plasma of pregnant compared with non-pregnant sows between Days 9 and 15 after oestrus (P<0.05, Table 6), while pregnenolone concentrations were similar (Table 6), it is proposed that pregnenolone sulphate in uterine fluid derives from the pool of pregnenolone sulphate in maternal plasma, rather than from the plasma pregnenolone pool. Furthermore, as there is no evidence to suggest that steroid production rates of pregnant and non-pregnant gilts differ between Days 9 and 12 after oestrus, the plasma pregnenolone sulphate values shown in Text-figure 6 indicate that the rate of clearance of this steroid sulphate from maternal plasma is higher in pregnant animals before Day 12 after oestrus. Precise mechanisms of transfer of steroids and/or steroid conjugates across the pig endometrium have not yet been elucidated.

Our own in-vitro studies have identified sulphokinase activity in intact pig blastocysts, and have shown that pregnenolone sulphate can be metabolized to pregnenolone and to progesterone by blastocysts cultured between Days 3 and 8 after fertilization (B. A. Stone & R. F. Seamark, unpublished data). If pregnenolone sulphate concentrated in uterine luminal fluids is metabolized to progesterone by pig blastocysts in vivo, this progesterone may be concentrated within the blastocoele cavity (Heap et al., 1981) and at the trophectoderm boundary layer where it can then provide
immune cytoprotection for the embryo (Siiteri et al., 1977; Fujisaka, Kawano, Haruyama & Mori, 1985) or, if released, stimulate secretory activity by the endometrium (Knight et al., 1973; Schlosnagle et al., 1974; Roberts et al., 1976; Adams, Bazer & Roberts, 1981; Pazleabas, Bazer & Roberts, 1982). Higher levels of progesterone in fluids recovered from pregnant tracts in the present study (P<0.05; Table 4) are consistent with progesterone synthesis and secretion by preimplantation pig blastocysts, although concentrations of progesterone in uterine fluid and in plasma of pregnant and of non-pregnant gilts were similar between Days 9 and 15 after oestrus (P>0.05; Table 6). Uterine metabolism of progesterone released by blastocysts is thus indicated, with no direct contribution by conceptus tissue to the maternal progesterone pool (see Robertson & King, 1974). Similarly, large quantities of progesterone which are synthesized by placental tissues of pigs later in gestation appear to be metabolized locally, and do not increase systemic progesterone levels (Kukoly, Knight & Notter, 1984).

Higher levels of progesterone in plasma of pregnant gilts at Day 15 after oestrus (P<0.05; Text-figure 6) are consistent with luteotrophic effects of oestrogens (Gardner, First & Casida, 1963; Bazer & Thatcher, 1977; Frank et al., 1978; Ford & Magness, 1980) which are secreted into the uterine lumen of pregnant sows at this time (Text-figure 4).

On the basis of these results, it is suggested that the high levels of the sulphoconjugates of pregnenolone and of DHEA in uterine fluid of the early pregnant pig serve as an important precursor pool for steroid synthesis by preimplantation pig embryos in vivo.
3.3 FREE AMINO ACIDS IN UTERINE FLUID

3.3.1 Introduction

A protein source is essential to blastocyst growth (e.g. Gwatkin & Meckley, 1965), and developmental responses to protein have been attributed to effects of free amino acids contributed by the protein source (Brinster, 1972). These amino acids include those which are essential to protein synthesis by blastocysts (Sellens, Stein & Sherman, 1981) and those which stimulate expansion of mammalian blastocysts in vitro (Daniel & Krishnan, 1967). Amino acids represent the most limiting group of nutrients in synthetic media used for culture of rabbit blastocysts in vitro (Kane & Foote, 1970).

In view of further evidence that endometrial secretion in sows is stimulated during early pregnancy (e.g. Knight, Bazer & Wallace, 1973), the following experiment compares free amino acid levels in uterine fluid collected from mated and un-mated gilts between Days 9 and 15 after oestrus. Further comparison is made to the free amino acid content of plasma.

3.3.2 Materials and methods

Animals

Uteri and blood samples were recovered from 12 Large White sows (6 un-mated and 6 mated) at slaughter, 9 to 15 days following the onset of the previous oestrus. All animals were fasted during the preceding 24 h. Following recovery of uteri (within 5 min of stunning and exsanguination), each uterine horn was cannulated above the bifurcation and flushed from the utero-tubal junction, toward the cannula (Stone et al., 1984 a), with 10 ml 0.9% saline at 4°C. Flushings of both uterine horns from each animal were pooled, transported in ice to the laboratory, and there centrifuged at
low speed (56 g, 20 min, 4°C) to precipitate conceptus tissue and/or other particulate matter. Supernatants were aliquoted and stored at -15°C. Precipitates from flushings of tracts of mated gilts were resuspended in 0.9% (w/v) saline and examined microscopically to confirm the presence of embryos consistent with the assumed stage of pregnancy (Anderson, 1978). Blood samples were collected from all animals at exsanguination and plasma aliquots stored at -15°C.

Amino acid analysis

Samples of plasma and uterine washings were deproteinized by picric acid addition, centrifugation and removal of excess acid with Dowex 2-X8 resin (Blackburn, 1968). Effluent from the columns was evaporated to dryness at 40°C on a rotary evaporator and the sample residues dissolved in pH 2.2 ion exchange buffer containing 400 nmol norleucine/ml. These solutions were chromatographed on an amino acid analyser (Locarte; London, W.12, England) using lithium buffers. Levels of 20 amino acids (11 essential and 9 non-essential) in the samples were determined by comparing chromatogram peak areas with respective areas for 24 amino acids in the external standard.

Statistics

For each amino acid measured, statistical differences between pregnant and non-pregnant values, and between plasma and histotroph data, were analysed by Student's t test (Clarke, 1969).

3.3.3 Results

The total contents of amino acids in uterine washings from pregnant and un-mated gilts are presented in Table 7. Concentrations of non-essential and essential free amino acids in plasma and in uterine fluid (corrected for
Table 7: Total content of essential and non-essential free amino acids (umol) in saline washings of gravid and of non-gravid uteri of gilts between Days 9 and 15 after oestrus/mating (mean ± s.e.m.; N = 6)

<table>
<thead>
<tr>
<th></th>
<th>GRAVID</th>
<th>NON-GRAVID</th>
<th>Significance of difference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>NON-ESSENTIAL</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ALANINE</td>
<td>17.58 ± 5.56</td>
<td>4.68 ± 1.31</td>
<td>*</td>
</tr>
<tr>
<td>ASPARTIC ACID</td>
<td>6.24 ± 2.70</td>
<td>2.48 ± 0.70</td>
<td>NS</td>
</tr>
<tr>
<td>CITRAMINE</td>
<td>0.88 ± 0.23</td>
<td>1.70 ± 0.99</td>
<td>NS</td>
</tr>
<tr>
<td>CYSTINE</td>
<td>1.40 ± 0.55</td>
<td>1.00 ± 0.49</td>
<td>NS</td>
</tr>
<tr>
<td>GLUTAMIC ACID</td>
<td>20.22 ± 6.64</td>
<td>33.44 ± 19.70</td>
<td>NS</td>
</tr>
<tr>
<td>GLYCINE</td>
<td>74.90 ± 28.49</td>
<td>14.02 ± 5.26</td>
<td>*</td>
</tr>
<tr>
<td>PROLINE</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SERINE</td>
<td>58.48 ± 27.70</td>
<td>3.32 ± 1.19</td>
<td>*</td>
</tr>
<tr>
<td>TYROSINE</td>
<td>2.84 ± 0.87</td>
<td>1.18 ± 0.32</td>
<td>*</td>
</tr>
<tr>
<td><strong>ESSENTIAL</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ARGinine</td>
<td>5.82 ± 1.30</td>
<td>0.46 ± 0.15</td>
<td>***</td>
</tr>
<tr>
<td>HISTIDINE</td>
<td>2.54 ± 0.82</td>
<td>0.38 ± 0.08</td>
<td>**</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>4.18 ± 1.40</td>
<td>1.16 ± 0.44</td>
<td>*</td>
</tr>
<tr>
<td>Leucine</td>
<td>7.52 ± 2.60</td>
<td>1.46 ± 0.42</td>
<td>**</td>
</tr>
<tr>
<td>LYSINE</td>
<td>6.16 ± 2.49</td>
<td>1.40 ± 0.42</td>
<td>*</td>
</tr>
<tr>
<td>METHIONINE</td>
<td>2.10 ± 0.67</td>
<td>0.56 ± 0.18</td>
<td>*</td>
</tr>
<tr>
<td>ORNITHINE</td>
<td>0.94 ± 0.29</td>
<td>0.70 ± 0.29</td>
<td>NS</td>
</tr>
<tr>
<td>PHENYLALANINE</td>
<td>2.66 ± 0.85</td>
<td>0.50 ± 0.11</td>
<td>**</td>
</tr>
<tr>
<td>THREONINE</td>
<td>7.80 ± 2.29</td>
<td>3.26 ± 1.02</td>
<td>NS</td>
</tr>
<tr>
<td>TRYPTOPHANE</td>
<td>9.96 ± 2.65</td>
<td>1.90 ± 0.73</td>
<td>**</td>
</tr>
<tr>
<td>VALINE</td>
<td>6.58 ± 2.28</td>
<td>1.66 ± 0.73</td>
<td>*</td>
</tr>
</tbody>
</table>

NS Not significant; * P < 0.05; ** P < 0.01; *** P < 0.001
flush volume) are shown in Tables 8 and 9 respectively.

The total content of 13 of the 20 amino acids measured in washings of pregnant uteri exceeded (P<0.05) respective non-pregnancy values (Table 7). Differences between levels of the other 7 amino acids in washings of pregnant and non-pregnant tracts were not significant (P>0.10).

3.3.4 Discussion

Enhanced accumulation of amino acids in fluids collected from the pregnant tracts (Table 7) is consistent with previous evidence for stimulation of transport of nutrients across the endometrium and for stimulation of endometrial secretion by conceptus tissues (Knight et al., 1973; Basha et al., 1980; Geisert et al., 1982 a).

Apart from dilution of arginine (P<0.01) and concentration of cystine (P<0.001) and aspartic acid (P<0.05), the results provide no evidence for modification of free amino acid concentrations in uterine fluid relative to plasma in non-pregnant gilts (Tables 8 and 9). By comparison, concentration of aspartic acid (P<0.05) and threonine (P<0.01) in uterine fluid from pregnant animals (Tables 2 and 3) and higher concentrations of serine and arginine in uterine fluid recovered from pregnant compared with non-pregnant tracts (P<0.01 and P<0.05 respectively; Tables 8 and 9) further indicates enhanced transport of amino acids across the endometrium in pregnant sows.

While there is currently no evidence for definitive roles for specific free amino acids in pre-implantation development of mammalian embryos, cysteine, tryptophane, phenylalanine, lysine, arginine and valine have been shown to be essential to cleavage of rabbit embryos beyond the 4-cell stage (Daniel & Olson, 1968), methionine, serine and threonine are essential to rabbit blastocyst growth in the absence of cofactors (Kane & Poote, 1970), and arginine and leucine appear to be critical to trophoblastic outgrowth in the mouse (Gwatkin, 1966). Furthermore, while the amino acid requirements for embryonic growth are dependent upon the stage of embryonic development
### Table 8: Concentrations (μM) of Non-Essential Free Amino Acids in Histotroph and in Plasma of Mated and Un-mated Sows Between Days 9 and 15 Post-Oestrus (Mean ± S.E.M.; N = 6)

<table>
<thead>
<tr>
<th></th>
<th>Alanine</th>
<th>Aspartic Acid</th>
<th>Citrulline</th>
<th>Cystine</th>
<th>Glutamic Acid</th>
<th>Glycine</th>
<th>Proline</th>
<th>Serine</th>
<th>Tyrosine</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Non-Pregnant</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Histotroph (H) (n=6)</td>
<td>374 ± 102</td>
<td>115 ± 23</td>
<td>93.8 ± 47</td>
<td>75.4 ± 14</td>
<td>1578 ± 892</td>
<td>1505 ± 329</td>
<td>-</td>
<td>161 ± 28</td>
<td>70.8 ± 32</td>
</tr>
<tr>
<td>Plasma (P) (n=6)</td>
<td>402 ± 37</td>
<td>28.6 ± 3</td>
<td>56.2 ± 11</td>
<td>1.2 ± 0.2</td>
<td>195 ± 20</td>
<td>628 ± 95</td>
<td>250 ± 27</td>
<td>98.4 ± 15</td>
<td>35.4 ± 2</td>
</tr>
<tr>
<td>Significance of difference</td>
<td><strong>NS</strong></td>
<td>*</td>
<td><strong>NS</strong></td>
<td><strong>NS</strong></td>
<td><strong>NS</strong></td>
<td><strong>NS</strong></td>
<td><strong>NS</strong></td>
<td><strong>NS</strong></td>
<td></td>
</tr>
<tr>
<td>H/P ratio</td>
<td>0.9</td>
<td>4.0</td>
<td>1.7</td>
<td>62.8</td>
<td>10.1</td>
<td>2.4</td>
<td>-</td>
<td>1.6</td>
<td>2.0</td>
</tr>
<tr>
<td><strong>Pregnant</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Histotroph (H) (n=6)</td>
<td>456 ± 177</td>
<td>105 ± 35</td>
<td>47.8 ± 5</td>
<td>26.5 ± 11</td>
<td>504 ± 152</td>
<td>1690 ± 318</td>
<td>-</td>
<td>1416 ± 404</td>
<td>68.6 ± 26</td>
</tr>
<tr>
<td>Plasma (P) (n=6)</td>
<td>332 ± 39</td>
<td>13.8 ± 2</td>
<td>65.0 ± 11</td>
<td>5.00 ± 1</td>
<td>109 ± 22</td>
<td>651 ± 112</td>
<td>280 ± 75</td>
<td>123 ± 28</td>
<td>51.4 ± 10</td>
</tr>
<tr>
<td>Significance of difference</td>
<td><strong>NS</strong></td>
<td>*</td>
<td><strong>NS</strong></td>
<td>*</td>
<td><strong>NS</strong></td>
<td><strong>NS</strong></td>
<td>-</td>
<td><strong>NS</strong></td>
<td></td>
</tr>
<tr>
<td>H/P ratio</td>
<td>1.4</td>
<td>7.6</td>
<td>0.7</td>
<td>5.3</td>
<td>4.6</td>
<td>2.6</td>
<td>-</td>
<td>11.6</td>
<td>1.3</td>
</tr>
<tr>
<td><strong>Histotroph</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Significance of difference between pregnant and non-pregnant concentrations</td>
<td><strong>NS</strong></td>
<td><strong>NS</strong></td>
<td><strong>NS</strong></td>
<td><strong>NS</strong></td>
<td><strong>NS</strong></td>
<td><strong>NS</strong></td>
<td><strong>NS</strong></td>
<td><strong>NS</strong></td>
<td><strong>NS</strong></td>
</tr>
<tr>
<td>Pregnant/Non-pregnant ratio</td>
<td>1.2</td>
<td>0.9</td>
<td>0.5</td>
<td>0.4</td>
<td>0.3</td>
<td>1.1</td>
<td>-</td>
<td>8.8</td>
<td>1.0</td>
</tr>
<tr>
<td><strong>Plasma</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Significance of difference between pregnant and non-pregnant concentrations</td>
<td><strong>NS</strong></td>
<td><strong>NS</strong></td>
<td><strong>NS</strong></td>
<td>*</td>
<td><strong>NS</strong></td>
<td><strong>NS</strong></td>
<td><strong>NS</strong></td>
<td><strong>NS</strong></td>
<td><strong>NS</strong></td>
</tr>
<tr>
<td>Pregnant/Non-pregnant ratio</td>
<td>0.8</td>
<td>0.5</td>
<td>1.2</td>
<td>4.2</td>
<td>0.6</td>
<td>1.0</td>
<td>1.2</td>
<td>1.2</td>
<td>1.5</td>
</tr>
</tbody>
</table>

NS: Not significant; *P < 0.05; **P < 0.01; ***P < 0.001; - not determined.
<table>
<thead>
<tr>
<th>Table 9: Concentrations (μM) of Essential Free Amino Acids in Histotroph and in Plasma of Mated and Un-mated Sows Between Days 9 and 15 Post-oestrus (Mean ± S.E.M.; N = 6)</th>
</tr>
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<td><strong>NON-PREGNANT</strong></td>
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<tr>
<td>Histotroph (H) (n=6)</td>
</tr>
<tr>
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<tr>
<td>Histidine</td>
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<td>Isoleucine</td>
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<td>Valine</td>
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<tr>
<td>Plasma (P) (n=6)</td>
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<td>Histidine</td>
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<td>Valine</td>
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<td>Significance of difference</td>
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<tr>
<td>Valine</td>
</tr>
<tr>
<td>Pregnant/Non-pregnant ratio</td>
</tr>
</tbody>
</table>

NS Not significant; *P < 0.05; **P < 0.01; ***P < 0.001; - not determined
(Daniel & Krishnan, 1967; Daniel & Olson, 1968), requirements for embryos of different species, but of the same developmental stage, may be similar (Gwatkin, 1966; Daniel & Olson, 1968). In this regard, our evidence for accumulation \( (P<0.05) \) of arginine, leucine, lysine, methionine, phenylalanine, serine, tryptophane and valine in gravid sow uteri between Days 9 and 15 after mating (Table 7) may be significant to normal pre-implantation development and trophoblastic outgrowth of pig embryos in vivo.

The predominant free amino acids in uterine washings of pregnant sows were glycine \( (1.69 \text{ mM}) \), serine \( (1.42 \text{ mM}) \), glutamic acid \( (0.50 \text{ mM}) \), alanine \( (0.46 \text{ mM}) \) and tryptophane \( (0.32 \text{ mM}; \text{Tables } 8 \text{ and } 9) \). Similarly, Iritani et al. (1974) found glycine to be the predominant amino acid in uterine fluid of dioestrous sows (Table 2), the concentration of glycine in that fluid \( (1.32 \text{ mM}) \) being similar to our determinations for non-pregnancy and pregnancy (Tables 8 and 9). The high level of glycine in histotroph from early pregnant sows is in accord with its abundance in plasma (about 0.65 mM, Table 8) and its assumed central metabolic role in mammalian blastocyst development (Kaye, 1983). Glutamic acid \( (1.98 \text{ mM}) \), glycine \( (1.5 \text{ mM}) \) and alanine \( (0.37 \text{ mM}) \) were predominant in uterine fluid of un-mated sows between Days 9 and 15 after coitus (Tables 8 and 9).

As the feed intakes of sows in this study were matched quantitatively and qualitatively, lower concentrations of aspartic acid and of glutamic acid in plasma of pregnant compared with non-pregnant gilts \( (P<0.01 \text{ and } P<0.05 \text{ respectively}; \text{Table } 8) \) indicate enhanced clearance of these amino acids during early pregnancy. Conversely, higher concentrations of cystine and methionine in the pregnancy samples \( (P<0.01 \text{ and } P<0.05 \text{ respectively}, \text{Tables } 8 \text{ and } 9) \) suggest reduced metabolic clearance of the sulphur-containing amino acids in pregnancy. Levels of the other free amino acids measured in plasma of pregnant and non-pregnant animals were similar (Tables 8 and 9).

Amino acid requirements for growth and development of pig blastocysts
have not previously been specified. This study defines levels of 20 amino acids in the uterine fluid which bathes pre-implantation pig blastocysts in vivo. How these levels reflect metabolism of specific free amino acids by pre-implantation blastocysts is not yet known, but the data establish a quantitative basis for amino acid supplementation of blastocyst culture media to provide an amino acid composition representative of the amino acid milieu in vivo. While high standard errors of means in the present study reflect wide between-animal variation in concentrations of certain amino acids in uterine fluid (Tables 8 and 9), the between-animal variance is small in comparison to the disparity between the amino acid composition of commercial culture media used in recent studies of pre-implantation development of pig embryos in vitro (e.g. Davis & Day, 1978; Lindner & Wright, 1978; Robl & Davis, 1981; Kuzan & Wright, 1982; Stone, Quinn & Seamark, 1984) and the mean composition of free uterine fluid (Table 10). In relation to those amino acids which have been shown to be essential to pre-implantation embryonic development in other mammalian species (arginine, cystine, leucine, lysine, methionine, phenylalanine, serine, tryptophane and valine), NCTC 135 medium (Evans, Bryant, Kerr & Schilling, 1964) provides an amino acid profile which most closely matches uterine fluid of early pregnant pigs (Table 10). In this respect, and in view of the necessity for inclusion of amino acids in synthetic media for culture of mammalian blastocysts in vitro (Kane & Foote, 1970), NCTC 135 can be recommended as a medium for maintenance of tissues of pre-implantation pig embryos in vitro.

Chromatograms of individual uterine washings in this study showed up to 30 peaks not associated with the 24 amino acids in the external standard. While these ninhydrin-positive compounds could not be identified, elucidation of their nature and origin(s) could prove significant to future understanding of specific amino acid requirements for growth of pig blastocysts in vitro and in vivo.
Table 10: The amino acid content of six commercial culture media, compared with the composition of free uterine fluid collected from uteri of pregnant gilts between Days 9 and 15 after mating (from Tables 8 and 9; um)

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Free uterine fluid</th>
<th>1*</th>
<th>2*</th>
<th>3*</th>
<th>4*</th>
<th>5*</th>
<th>6*</th>
</tr>
</thead>
<tbody>
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<td>ALANINE</td>
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<td>-</td>
<td>-</td>
<td>101</td>
<td>100</td>
<td>561</td>
<td>353</td>
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<td>ASPARTIC ACID</td>
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<td>-</td>
<td>-</td>
<td>200</td>
<td>200</td>
<td>400</td>
<td>127</td>
</tr>
<tr>
<td>CITRAGINE</td>
<td>48</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CYSTINE/CYSTEINE</td>
<td>27</td>
<td>500</td>
<td>130</td>
<td>100</td>
<td>100</td>
<td>84</td>
<td>44</td>
</tr>
<tr>
<td>GLUTAMIC ACID/GLUTAMINE</td>
<td>504</td>
<td>2000</td>
<td>2000</td>
<td>1100</td>
<td>1100</td>
<td>1710</td>
<td>985</td>
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<tr>
<td>GLYCINE</td>
<td>1690</td>
<td>-</td>
<td>-</td>
<td>100</td>
<td>100</td>
<td>666</td>
<td>180</td>
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<td>PROLINE</td>
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<td>-</td>
<td>-</td>
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<td>300</td>
<td>435</td>
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<td>SERINE</td>
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<td>-</td>
<td>100</td>
<td>100</td>
<td>476</td>
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<td>30</td>
<td>221</td>
<td>91</td>
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<td>600</td>
<td>1000</td>
<td>1000</td>
<td>332</td>
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<td>HISTIDINE</td>
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<td>1030</td>
<td>200</td>
<td>100</td>
<td>100</td>
<td>141</td>
<td>172</td>
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<td>Isoleucine</td>
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<td>200</td>
<td>400</td>
<td>20</td>
<td>30</td>
<td>305</td>
<td>138</td>
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<tr>
<td>LEUCINE</td>
<td>173</td>
<td>1000</td>
<td>400</td>
<td>99</td>
<td>100</td>
<td>915</td>
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<td>LYSINE</td>
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<td>160</td>
<td>200</td>
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<td>METHIONINE</td>
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<td>Threonine</td>
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<td>30</td>
<td>100</td>
<td>504</td>
<td>159</td>
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<td>50</td>
<td>3</td>
<td>10</td>
<td>98</td>
<td>86</td>
</tr>
<tr>
<td>VALINE</td>
<td>121</td>
<td>1000</td>
<td>400</td>
<td>30</td>
<td>100</td>
<td>427</td>
<td>213</td>
</tr>
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</table>

*1. Eagle's basal medium with amino acids (spindle, 1980)  
2. MEM, Earle's powder (Eagle, 1959)  
3. Ham's nutrient mixture F-10 (Ham, 1963)  
4. Ham's nutrient mixture F-12 (Ham, 1965)  
5. Medium 199 (Morgan, Morton & Parker, 1950)  
6. NCTC 135 medium (Evans, Bryant, Kerr & Schilling, 1964)
3.4 SUMMARY

It is postulated that the endometrium of the pig concentrates steroid hormones in histotroph between Days 9 and 15 after oestrus and that pregnancy-associated increases in steroid levels in uterine fluids between Days 9 and 15 after mating reflect steroidogenesis by embryonic tissues and modification of enzyme activities within uterine tissues under the influence of progestagens.

The pool of steroid sulfoconjugates (pregnenolone sulphate and DHEA sulphate) concentrated in uterine fluid between Days 9 and 15 after mating (Table 5) could serve as an important precursor source for progestagen, androgen and oestrogen synthesis by tissues of pig embryos prior to implantation (Chapter 2.5.3.1).

These steroids (in particular progesterone and oestradiol) can then stimulate endometrial transport and exocytosis of potential growth factors (see Chapters 2.3 and 3.2) and, in this respect, the total content of 13 of the 20 amino acids measured in washings of uteri of pregnant sows in this study exceeded (P<0.05) respective non-pregnancy values. Concentrations of aspartic acid, glutamic acid, glycine, serine and threonine were also higher (P<0.05) in free uterine fluid relative to plasma in the pregnant sows, the predominant free amino acid in uterine fluid collected from these animals being glycine. Previous studies have established that 8 of the amino acids accumulated in the fluids of gravid uteri (arginine, leucine, lysine, methionine, phenylalanine, serine, tryptophane and valine; Table 7) are essential to in vitro development of blastocysts of other mammals (mouse and rabbit; see Chapter 3.3).

The occurrence of steroid hormones in uterine fluid, and their metabolism by embryonic tissues, may thus prove to be essential to early embryonic development in sows. Furthermore, an element of the essentiality of the ovaries in the maintenance of pregnancy (Short, 1956; du Mesnil du Buisson & Dauzier, 1957; First & Staigmiller, 1973; Nara, Darmadja & First,
1981) may then be the provision of those steroids (or precursors to the steroids) which accumulate in uterine fluid of sows during early pregnancy (Table 4). As ovarian tissues appear to be the principal source of pregnancy-related steroid hormones in sows (see Chapter 2.5.3.1), the experiments described in the following chapter have been designed to quantitate the contribution of these tissues to maternal plasma steroid pools. It is assumed that histotroph steroids (or their precursors) derive from plasma.

The results of the present chapter indicate wide variation in total levels, and in concentrations, of steroid hormones and free amino acids in uterine fluids of pigs between Days 9 and 15 after oestrus/coitus. While part of this variance will be attributable to between-day changes in composition of histotroph (see Text-figures 4 and 5), levels of components of this milieu also vary widely within days (ibid). Optimum levels of particular steroids or free amino acids for development of preimplantation pig blastocysts in vivo are not yet established. However, whatever these values are, statistical variation within the present data shows that most embryos are likely to be bathed in histotroph which provides suboptimal levels of these components. Heterogeneity in the composition of histotroph may thus, itself, be an important contributing factor to high levels of preimplantation embryonic mortality in the pig. This Thesis does not further pursue this specific likelihood in detail.
CHAPTER IV

ORIGINS OF STEROIDS ACCUMULATED IN UTERINE FLUID DURING THE PRE-IMPLANTATION PHASE OF PREGNANCY

4.1 STEROIDS OF OVARIAN ORIGIN

4.1.1 Introduction

Peripheral levels of steroids are determined by endogenous rates of steroid production (Broom, Johnson, Phillipou & Seamark, 1981). Suppression or removal of a steroid source will, therefore, be associated with diminished levels of those steroids in plasma. Depressed peripheral levels of steroids in gilts following ovariectomy can thus confirm contribution by the ovary to levels of those steroids in plasma of intact animals, and relative changes in plasma levels of particular steroids following ovariectomy can reflect the relative contribution of the ovary to plasma pools of these steroids, or their precursors.

In order to determine which plasma steroids are derived from ovaries in intact gilts, and to estimate the relative contribution of the ovaries to plasma pools of these steroids between Days 9 and 15 after oestrus, levels of steroids have been determined in peripheral plasma of oophorectomized gilts, and in plasma of unmated gilts, between Days 9 and 15 after oestrus.

To further confirm ovarian origin of those steroids which are present in plasma at diminished levels following ovariectomy, levels of the same steroids are investigated in peripheral plasma and in ovarian venous plasma of unmated gilts during the late luteal phase of the oestrous cycle. For comparison with pregnant animals, these same values are determined in pregnant sows between Days 9 and 17 after mating.
4.1.2 Effect of oophorectomy on plasma steroid content

Materials and methods

Anaesthesia was induced in four sexually mature gilts by intravenous (peripheral ear vein) infusion of a solution of sodium thiopentone (Abbott Australasia Pty. Ltd., Sydney, Australia) in sterile water, to provide 1.0 g sodium thiopentone per 90 kg body weight. Anaesthesia was maintained by closed circuit general anaesthesia (CIG Midget 3 anaesthetic apparatus; Medishield Ramsay, Kent Town, South Australia), ventilating with 3 to 6 l per hour of 2.0% halothane (Fluothane; ICI Australia Operations Pty. Ltd., Villawood, N.S.W.) in medical grade oxygen:nitrous oxide (2:1). The anaesthetized gilts were bilaterally ovariectomized following mid-ventral laparotomy. Two blood samples were collected from each animal (jugular puncture) during the second week after ovariectomy. Following centrifugation (2011 g, 20 min, 4°C), plasma aliquots were aspirated and stored at -15°C.

The collection and preparation of plasma samples from intact gilts between Days 9 and 15 after oestrus (N=16) has been described previously (Chapter 3.2.2.1), as have the appropriate techniques for extraction, chromatography and radioimmunoassay of steroids in sow plasma (Chapter 3.2.2.2).

For each steroid measured, differences between oophorectomized and intact gilt data were statistically analysed using Student's t-test (Clarke, 1969).

Results

Levels (nm; mean ± s.e.m.) of steroids in plasma of oophorectomized gilts, and of intact gilts between Days 9 and 15 after oestrus, are presented in Table 11.
Table 11: Levels of steroids (nM; mean ± s.e.m.) in plasma of oophorectomized gilts (Ox; N=8) in comparison with levels in plasma of intact gilts (Ov; N=16) between Days 9 and 15 after oestrus.

<table>
<thead>
<tr>
<th>Steroid</th>
<th>Oophorectomized (Ox)</th>
<th>Intact (Ov)+</th>
<th>Significance of difference</th>
<th>(Ov-Ox x 100)Φ†</th>
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<tbody>
<tr>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>C21</td>
<td></td>
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</tr>
<tr>
<td>Pregnenolone</td>
<td>2.70 ± 0.43</td>
<td>3.36 ± 0.99</td>
<td>NS</td>
<td>19.6</td>
</tr>
<tr>
<td>Pregnenolone sulphate</td>
<td>0.10 ± 0.05</td>
<td>0.10 ± 0.02</td>
<td>NS</td>
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<td>Progesterone</td>
<td>3.17 ± 0.33</td>
<td>72.7 ± 12.0</td>
<td>**</td>
<td>99.8</td>
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<td>C19</td>
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<tr>
<td>DHEA</td>
<td>0.16 ± 0.05</td>
<td>0.10 ± 0.02</td>
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<td>0.0</td>
</tr>
<tr>
<td>DHEA sulphate</td>
<td>0.50 ± 0.29</td>
<td>0.61 ± 0.10</td>
<td>NS</td>
<td>18.0</td>
</tr>
<tr>
<td>Androstenedione</td>
<td>0.40 ± 0.11</td>
<td>0.45 ± 0.04</td>
<td>NS</td>
<td>11.1</td>
</tr>
<tr>
<td>Testosterone</td>
<td>0.063 ± 0.03</td>
<td>0.85 ± 0.18</td>
<td>*</td>
<td>91.6</td>
</tr>
<tr>
<td>C18</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oestrone</td>
<td>0.04 ± 0.01</td>
<td>0.05 ± 0.01</td>
<td>**</td>
<td>20.0</td>
</tr>
<tr>
<td>Oestrone sulphate</td>
<td>0.10 ± 0.01</td>
<td>0.32 ± 0.04</td>
<td>**</td>
<td>68.8</td>
</tr>
<tr>
<td>Oestradiol</td>
<td>0.017 ± 0.010</td>
<td>0.03 ± 0.01</td>
<td>NS</td>
<td>44.0</td>
</tr>
</tbody>
</table>

† - Estimated contribution of the ovaries to levels of each steroid in plasma of unmated gilts, between Days 9 and 15 after oestrus (as a percentage).
The relative contributions of ovaries to levels of each steroid in plasma of intact gilts were estimated by quantitating the depression in plasma steroid levels in oophorectomized gilts compared to levels in the intact gilts. The calculated values are presented in Table 11.

4.1.3 Levels of steroids in ovarian venous plasma of mated and un-mated gilts between Days 9 and 15 after oestrus

Materials and methods

Cuffed indwelling cannulae (Dow Corning Silastic Medical Grade Tubing; cat. # 602-285, Dow Corning Corporation, Michigan, U.S.A.; 1.58 mm ID, 3.18 mm OD) were implanted into an ovarian vein, via a uterine branch, in each of 5 (2 mated, 3 un-mated) anaesthetized (Chapter 4.1.2) sows on Days 7 or 8 after the onset of oestrus. Cannulae were maintained by twice daily flushing with sterile saline (0.9%, w/v) containing heparin (100 i.u./ml; Heparin sodium, Glaxo Australia Pty. Ltd., Boronia, Victoria, Australia).

Following recovery, blood samples were collected daily from patent cannulae between Days 9 and 15 after oestrus. Blood could not be drawn from the ovarian vein of one un-mated gilt after Day 13 post oestrus, nor from one mated gilt after Day 12 post coitum.

Levels of pregnenolone, pregnenolone sulphate, progesterone, DHEA, DHEA sulphate, androstenedione, testosterone, oestrone, oestrone sulphate and oestradiol were determined in chromatographed extracts of ovarian venous plasma, according to the methods detailed previously (Chapter 3.2).

Results

Levels (nM; mean ± s.e.m.) of steroids in ovarian venous plasma from mated and un-mated animals are compared with respective peripheral steroid levels (from Table 5) in Table 12.
Table 12: Steroid hormone content (nM; mean ± s.e.m.) of ovarian venous plasma, and of peripheral (external jugular) plasma, of mated and of unmated gilts between Days 9 and 17 after oestrus.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>C21</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pregnenolone</td>
<td>47.54 ± 8.90</td>
<td>9.90 ± 2.34</td>
<td>3.88 ± 0.84</td>
<td>3.36 ± 0.99</td>
</tr>
<tr>
<td>Pregnenolone sulphate</td>
<td>124.8 ± 10.5</td>
<td>56.5 ± 18.0</td>
<td>25.0 ± 1.9</td>
<td>37.3 ± 8.4</td>
</tr>
<tr>
<td>Progesterone</td>
<td>409.9 ± 25.4</td>
<td>95.1 ± 31.9</td>
<td>89.2 ± 6.4</td>
<td>72.7 ± 12.0</td>
</tr>
<tr>
<td><strong>C19</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DHEA</td>
<td>0.60 ± 0.08</td>
<td>0.19 ± 0.02</td>
<td>0.44 ± 0.10</td>
<td>0.10 ± 0.02</td>
</tr>
<tr>
<td>DHEA sulphate</td>
<td>1.21 ± 0.18</td>
<td>0.87 ± 0.13</td>
<td>1.34 ± 0.27</td>
<td>0.61 ± 0.10</td>
</tr>
<tr>
<td>Androstenedione</td>
<td>8.04 ± 0.83</td>
<td>1.35 ± 0.34</td>
<td>1.15 ± 0.14</td>
<td>0.45 ± 0.04</td>
</tr>
<tr>
<td>Testosterone</td>
<td>2.97 ± 0.39</td>
<td>0.21 ± 0.09</td>
<td>0.84 ± 0.16</td>
<td>0.85 ± 0.18</td>
</tr>
<tr>
<td><strong>C18</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oestrone</td>
<td>0.22 ± 0.04</td>
<td>&lt;0.10</td>
<td>0.10 ± 0.05</td>
<td>0.05 ± 0.01</td>
</tr>
<tr>
<td>Oestrone sulphate</td>
<td>0.69 ± 0.10</td>
<td>0.11 ± 0.03</td>
<td>0.68 ± 0.16</td>
<td>0.32 ± 0.04</td>
</tr>
<tr>
<td>Oestradiol</td>
<td>0.42 ± 0.12</td>
<td>0.04 ± 0.01</td>
<td>0.03 ± 0.01</td>
<td>0.03 ± 0.01</td>
</tr>
</tbody>
</table>

+ - From Table 5.
4.1.4 Discussion

Levels of pregnenolone sulphate, progesterone, testosterone and oestrone sulphate in plasma of oophorectomized gilts were lower ($P<0.05$) than respective levels in plasma of un-mated gilts between Days 9 and 15 after oestrus (Table 11), indicating that these steroids, and/or their precursors, are of ovarian origin in gilts during the luteal phase of the oestrous cycle. Differences in steroid concentrations between peripheral and ovarian venous plasma in un-mated gilts (Table 12) are consistent with ovarian origin of progesterone and pregnenolone sulphate, but average concentrations of testosterone and oestrone sulphate in ovarian venous plasma of un-mated animals tend to be lower than the respective peripheral levels (Table 12). This evidence suggests that the higher concentrations of testosterone and oestrone sulphate in peripheral plasma of intact, unmated, gilts between Days 9 and 15 after oestrus (Table 11) may be attributable to release of precursors to those steroids by the ovaries of intact cycling animals. Levels of pregnenolone and androstenedione in ovarian and in peripheral venous plasma of un-mated gilts (Table 12) indicate their ovarian origin, although levels of these steroids in plasma of oophorectomized gilts (2.70 and 0.40 nM respectively) were not significantly lower than intact gilt values (3.36 and 0.45 nM respectively; $P>0.10$; Table 11). The steroid data presented in Tables 11 and 12 are consistent with peripheral conversion of ovarian-derived androstenedione to testosterone and/or oestrone sulphate, and conversion of ovarian-derived pregnenolone to pregnenolone sulphate.

By comparing steroid concentrations in ovarian venous and in peripheral venous plasma of mated and un-mated gilts (Table 12), ovarian secretion of progestagens, androstenedione and oestradiol appears to be enhanced during pregnancy. This finding is in accord with luteotrophic effects of conceptus tissues during early pregnancy in this species (Perry et al., 1976), and is investigated further in the following Section.
4.2 DETERMINATION OF ENDOGENOUS RATES OF PRODUCTION AND CLEARANCE OF
PROGESTERONE IN EARLY-PREGNANT, AND IN UN-MATED, GILTS

4.2.1 Introduction

The previous results showed that pregnenolone, pregnenolone sulphate, progesterone, DHEA, androstenedione, testosterone and oestradiol can be released by ovarian tissues, to appear at elevated levels in the ovarian venous drainage (Table 12). Furthermore, the predominant steroids in peripheral venous plasma of mated and un-mated gilts between Days 9 and 15 after oestrus (progesterone and pregnenolone sulphate; see Table 5) have been shown to originate principally from ovarian tissues (Table 11; Erb et al., 1962; Nara, Darmadja & First, 1981). However, despite the implied central metabolic role for progestagens in early pregnancy (e.g. Diehl & Day, 1974; Chapter 2.5.2), and three decades of experiments which have employed ovariectomized sows in which pregnancy is maintained by exogenous progesterone treatment (Table 3), endogenous rates of production of progestagens and other ovarian steroids have not previously been determined in this species. As a basis to studies on modification of maternal endocrine status to reduce levels of early embryonic mortality, the present study determines endogenous production rates (PR) and metabolic clearance rates (MCR; the volume of plasma which is completely and irreversibly cleared of steroid in unit time; Tait, 1963) of progesterone in sows between Days 11 and 17 post coitum, during which period progesterone is the predominant ovarian steroid (Heap, 1972). For comparison, PRs and MCRs have also been determined for un-mated sows between Days 11 and 17 after oestrus.

In view of ethical problems associated with determination of steroid PRs and MCRs by constant infusion of radioactive tracers (e.g. Lin, Lin, Erlenmeyer, Kline, Underwood, Billiar & Little, 1972), tracer steroids used
in the present study are labelled with a non-radioactive isotope (deuterium). Other advantages in using deuterium-labelled steroids as tracers in biological systems have been discussed previously (Seamark, Phillipou & McIntosh, 1977; Johnson, Phillipou, Blair & Seamark, 1979; Johnson, Phillipou & Seamark, 1981).

4.2.2 Materials and Methods

Animals

Fourteen Large-White sows (9 mated and 5 un-mated; 100-130 kg liveweight), between Days 9 and 13 after oestrus, were retained from the breeding herd at the Northfield Pig Research Unit (NPRU), Department of Agriculture, South Australia.

Intra-venous cannulation

Animals were anaesthetized (Chapter 4.1.2) and, using standard sterile technique, indwelling teflon cannulae (Cole-Palmer Instrument Co., Edwards Laboratories, Australia) were surgically implanted into the left cephalic (Takken & Williams, 1981) and right maxillary (Christison & Curtin, 1969) veins of each. Prior to insertion, the lumen of each cannula was filled with sterile saline (0.9%, w/v) containing heparin (100 i.u/ml; Heparin sodium, Glaxo Australia Pty. Ltd., Boronia, Victoria, Australia) and cidozylocin (0.16 mg/ml; Gentamicin sulphate, Roussel Pharmaceuticals Pty. Ltd., Castle Hill, N.S.W.). Cannulae were flushed four times with this solution during the 48-h recovery period.

Synthesis of tracer

The method followed for preparation of 7,7,16-\textsuperscript{d_{3}} progesterone has been
detailed by this laboratory previously (Johnson, Phillipou & Seamark, 1981).

Infusion

Following recovery of sows from surgery, solutions of 7,7,16-d₃ progesterone in 0.9% saline, containing plasma (10% v/v) from the same subject, were infused for 3.5 h via the cephalic vein cannulae (Constant Injection Apparatus, Palmer, England) at constant rates (near 14 nmoles/min). During infusion, blood samples were drawn half-hourly from the indwelling maxillary vein cannulae. Blood samples were centrifuged and aliquots of plasma stored at -15°C.

At the termination of each infusion, cephalic vein infusion cannulae were withdrawn and aliquots of infusate collected from the distal end of each were stored at -15°C.

Progesterone analysis

Levels of deuterated and endogenous progesterone in aliquots of infusate and in plasma were determined by gas chromatography/mass spectrometry (Johnson, Broom, Cox, Phillipou & Seamark, 1983) and PRs and MCRs of progesterone were calculated from these values (Tait, 1963; Johnson, Broom, Cox, Matthews, Phillipou & Seamark, 1983). At post mortem, about 10 days after each infusion, numbers of CL were recorded, and pregnancy in mated sows was confirmed.

Statistics

Differences in mean peripheral progesterone levels (PP), PR and MCR values between pregnant and non-pregnant sows were analysed using Student's t test (Clarke, 1969). Correlations between PP, PR, MCR and Days post oestrus/post
coitus; and between PP, PR and MCR, were examined by least-squares linear regression analysis (Snedecor & Cochran, 1980).

4.2.3 Results

Progesterone PR, MCR and PP values, during the period 11 to 17 days post oestrus/coitus, are shown in Text-figures 7, 8 and 9 respectively. Mean progesterone PR was higher (P<0.01) in pregnant (274 ± 26 [s.e.m.] umoles/24 h) than in non-pregnant (158 ± 12 umoles/24 h) gilts. Circulating PP levels were also higher (P<0.05) during pregnancy (86 ± 9 nM compared with 59 ± 3 nM in un-mated animals), but mean MCRs (3348 ± 312 and 2735 ± 275 1/24 h respectively) were similar (P>0.10).

MCRs and PPs of progesterone were significantly (P<0.05) correlated with the number of days gestation in the pregnant animals. Other regressions with Days post oestrus/coitus were not statistically significant.

Of the correlations between PR, MCR and PP values, only the regression between PP and MCR in pregnant animals was significant (PP = [141.9 - (0.017xMCR)]; r = 0.61, P<0.1; Text-figure 10). Neither PP nor PR values were significantly correlated with numbers of CL.

Endogenous rates of progesterone production in individual sows (mated and un-mated) in this study ranged between 464.6 and 123.4 umoles/24 h. The rate of infusion of deuterated progesterone in all sows (near 14 nmoles/min) was thus <17% of the endogenous PR, and we propose that infusion of deuterated progesterone at this rate would not significantly perturb endogenous progesterone dynamics. In this respect, the determinations of PR and MCR of progesterone would be, at the worst, 17% lower than the true endogenous rates.
Text-figure 7: Rates of endogenous progesterone production (umol/24 h) by mated (●) and unmated (○) gilts between Days 11 and 17 after oestrus.
Text-figure 8: Rates of metabolic clearance of progesterone (thousand litres/24 h) by mated (●) and unmated (○) gilts between Days 11 and 17 after oestrus.
Text-figure 9: Levels of progesterone in peripheral plasma (nM) of mated (●) and unmated (○) gilts between Days 11 and 17 after oestrus.
Text-figure 10: Inverse correlation (P<0.05) between peripheral plasma progesterone levels (nM) and metabolic clearance rates of progesterone (thousand litres/24 h) in pregnant gilts between Days 11 and 17 after mating.
4.2.4 Discussion

The increasing MCR of progesterone in pregnant gilts after Days 11-12 of gestation ($P<0.05$; Text-figure 8) is consistent with clearance of progesterone from the larger volume of blood directed toward the endometrial stroma in pregnant sows on Days 13 and 15 after mating, compared with Day 11 after mating (Ford, Christenson & Ford, 1982). The highest mass clearance of progesterone (MCR x PP) in pregnant sows in the present study was near 20 umoles/h on Day 14 after mating (calculated from data presented in Text-figures 8 and 9). In this regard, the high level of sulphotransferase activity in endometrial tissues of pregnant sows at this same time (Dwyer & Robertson, 1980) is stimulated by progesterone (Pack et al., 1979; Meyers et al., 1983) and effects sulphoconjugation of conceptus-derived oestrogen prior to its release to the maternal circulation as a luteotrophin (Gardner, First & Casida, 1963; Perry et al., 1976; Bazer & Thatcher, 1977; Frank et al., 1978; Ford & Magness, 1980). Stimulation of endometrial sulphotransferase activity by progesterone also provides a mechanism for accumulation of steroid sulphoconjugates in histotroph (Chapter 3.2) and may thus prove to be essential to the maintenance of conceptus tissues during early pregnancy in sows (Chapter 2.5.2). This and other progesterone-dependent functions in early pregnancy may justify further studies on correlations between the mass clearance of progesterone (MCR x PP) and early embryonic viability/mortality. For example, treatment of sows with 80-160 umoles progesterone daily during early pregnancy has been shown to be associated with increased litter size (Wildt et al., 1976), and there is a positive correlation between rates of urinary excretion of progesterone metabolites and early embryonic survival in early pregnant sows (Glasgow, Mayer & Dickerson, 1951; Mayer, Glasgow & Gawienowski, 1961). In sheep, exclusion of either progesterone or oestradiol from steroid replacement regimes in ovariectomized pregnant animals is associated with increased mortality of embryos, and with growth retardation of surviving embryos.
(Miller, Moore, Murphy & Stone, 1977; Moore & Miller, 1982).

Uterine arterial blood-flow in pregnant and non-pregnant sows between Days 11 and 15 of gestation are similar (Ford, Christenson & Ford, 1982), and the results of our study show no difference in the MCRs of progesterone between pregnant and un-mated sows during the same period (Text-figure 8). Similarly, progesterone MCRs are not altered during pregnancy in women (Little, Tait, Tait & Erlenmeyer, 1966; Lin et al., 1972) or in rats (Pepe & Rothchild, 1973).

Progesterone PR was higher (P<0.01) in pregnant (273 umoles/24 h) than in non-pregnant (158 umoles/24 h) animals in the present study. This finding supports previous evidence that secretions of blastocysts and/or of the endometrium of the gravid uterus after Day 10 of pregnancy are luteotrophic (Frank et al., 1978; Kidder, Casida & Grummer, 1955; Gardner, First & Casida, 1963) and is consistent with evidence for enhanced progesterone production during pregnancy in other species (e.g. Pepe & Rothchild, 1973).

The results also indicate that the differences in circulating progesterone levels between pregnant and un-mated pigs (Days 11-17 post oestrus/coitus; P<0.05; Text-figure 9) are attributable to an increased production of progesterone in the pregnant animals (P<0.01; Text-figure 7), rather than to decreased progesterone clearance. Similarly, levels of progesterone in peripheral plasma of women are correlated with endogenous rates of progesterone production (Broom et al., 1981). Within the pregnant group of gilts, declining plasma progesterone levels between Days 11 and 17 after coitus were associated with increasing MCR (P<0.05; Text-figure 8), and not with decreasing progesterone PR.

CL are the principal source of progesterone in sows (Erb et al., 1962; Nara, Darmadja & First, 1981), in accord with a >95% depression in plasma progesterone levels following oophorectomy (Table 11). The progesterone PRs reported will thus closely approximate ovarian progesterone PRs and provide a quantitative basis for physiological substitution of this progestagen in
ovariectomized sows between Days 11 and 17 of pregnancy. Results from studies in which progesterone is supplemented, or replaced, to within these ranges can then be related to normal physiological states. In this regard, most of the progesterone substitution and supplementation protocols to which early pregnant sows have previously been subjected (Chapter 2.5) are at variance to the physiological ranges determined in the present study (202-464 μmol/24 h). In relation to elucidation of roles for progesterone in early pregnancies of intact animals, conclusions drawn from the earlier studies are thus speculative.
4.3 ESTIMATION OF ENDOGENOUS RATES OF RELEASE OF STEROIDS (OTHER THAN PROGESTERONE) BY OVARIES OF MATED AND UNMATED GILTS BETWEEN DAYS 9 AND 17 AFTER OESTRUS

4.3.1 Introduction

Results from the previous experiment provide a physiological basis for progesterone substitution in ovariectomized gilts. However, while progesterone appears to be the principal steroidogenic product of luteal tissues in mated and unmated sows between Days 11 and 17 after oestrus (Heap, 1972), sow ovaries also release pregnenolone, pregnenolone sulphate, DHEA, androstenedione, testosterone and oestradiol during the luteal phase of the oestrous cycle (Table 12). These steroids should thus also be considered for inclusion in steroid cocktails formulated to authentically replace ovarian endocrine secretion in ovariectomized gilts. As a basis to this replacement, the experiment described below approximates endogenous rates of production of these steroids in mated and in un-mated gilts between Days 9 and 17 after the onset of oestrus.

4.3.2 Background

For a uniquely ovarian product 'a' which is secreted into the vasculature, the effective rate of secretion of 'a' will be a function of the difference in content of 'a' between the ovarian arterial supply and the ovarian venous drainage.

Simply,

\[ PR_a = (F \times [V_a - A_a]) \]

where;

- \( PR_a \): endogenous rate of release of 'a' (nmole/24 h)
V_a \quad \text{concentration of 'a' in the ovarian venous drainage (nM)}

A_a \quad \text{concentration of 'a' in the ovarian arterial supply (nM)}

F \quad \text{a blood-flow constant (1/24 h)}

If PR, V and A are then determined for a discrete ovarian product, 'F' can be calculated and used in formulae which can approximate rates of ovarian secretion of other blood-borne compounds. While the value of 'F' will be in part determined by ovarian blood-flow, it will not equal ovarian blood-flow unless 'V' is determined for ovarian venous blood collected at its immediate exit from the ovary, prior to its admixture with venous plasma from tributaries in and below the ovarian pedicle. That is, for a uniquely ovarian product 'a', 'V_a' will diminish and the calculated value 'F' will increase in approximate proportion to the anatomical dislocation between the ovary and the point of venous sampling. Regardless, where a set of ovarian venous blood samples have been collected from the same cannula, or from a number of cannulae which have been implanted at a common anatomical site in animals of matched size and reproductive status, the degree of dilution of all ovarian products in blood samples drawn from those cannulae will be similar. Provided all above conditions are met, an 'F' value calculated from 'PR_a', 'V_a' and 'A_a' determinations (for a unique ovarian product 'a') will be a valid constant in the formula,

\[ PR_b = (F \times [V_b - A_b]) \]

In this regard, progesterone is a predominantly (>95%, Table 11) ovarian product which is secreted toward the ovarian vein (Table 12). Furthermore, the endogenous production rates of progesterone in mated and in unmated gilts between Days 11 and 17 after oestrus are established (Chapter 4.2; Text-figure 7), as are the respective concentrations of progesterone in the ovarian venous drainage (Table 12). If it is further assumed that the progesterone content of plasma is not significantly altered in the pulmonary
circulation, then the concentration of progesterone in the external jugular vein will approximate 'A'. Transforming the 'PR' formula, and substituting concentrations of progesterone in the external jugular vein and in the ovarian vein (Table 12) for 'A' and 'V' respectively, values of 'F' for mated and un-mated gilts between Days 11 and 17 post oestrus can be calculated.

\[ F = \frac{PR}{[V - A]} \]

1/24 h.

4.3.3 Calculation of 'F' values on the basis of the progesterone data

**Mated gilts, 11-17 days after coitus**

\[ PR = 274000 \quad \text{(nmoles/24 h ; see Chapter 4.2)}, \]
\[ V = 409.9 \quad \text{(nM ; see Table 12)}, \]
\[ A = 89.2 \quad \text{(nM ; see Table 12)}. \]
\[ F = \frac{274000}{[409.9 - 89.2]} \]
\[ = 854 \quad 1/24 \text{ h} \]

**Un-mated gilts, 11-17 days after oestrus**

\[ PR = 158000 \quad \text{(nmoles/24 h ; see Chapter 4.2)}, \]
\[ V = 95.1 \quad \text{(nM ; see Table 12)}, \]
\[ A = 72.7 \quad \text{(nM ; see Table 12)}. \]
\[ F = \frac{158000}{[95.1 - 72.7]} \]
\[ = 7053 \quad 1/24 \text{ h} \]

Using the above estimates of 'F', and substituting the appropriate steroid concentrations for 'A' and 'V' in the 'PR' formula, endogenous rates of secretion of pregnenolone, pregnenolone sulphate, DHEA, androstenedione, testosterone and oestradiol by ovaries of mated and un-mated gilts (Days 11
to 17 after oestrus) can be approximated.

4.3.4 Results

Ovarian production rates (PR) of pregnenolone, pregnenolone sulphate, DHEA, androstenedione, testosterone and oestradiol, estimated according to,

\[ PR = (F \times [V - A]) \quad \text{nmoles/24 h} \]

are presented in Tables 13 and 14.

4.3.5 Discussion

While these results confirm earlier indications that progesterone is the predominant steroid product of ovaries of sows during the luteal phase of the oestrous cycle, or during early pregnancy (Heap, 1972; Table 12), significant quantities of pregnenolone sulphate are also released (Tables 13 and 14). Ovarian-derived pregnenolone sulphate is not obligatory to maintenance of pregnancy in ovariectomized gilts (pregnancy can be maintained with progesterone therapy alone; see Table 3) but ovaries appear to provide >99% of the plasma pool of this steroid during the luteal phase of the oestrous cycle (Table 11) and pregnenolone sulphate accumulated in uterine fluid (Table 4) appears to derive from this maternal plasma pool (Chapter 3.2.4). Metabolic roles and requirements for this steroid conjugate should therefore be considered where pregnancy is being maintained in ovariectomized sows by steroid treatment. In this respect, a later experiment examines the potential for metabolism of pregnenolone sulphate by pre-implantation pig embryos (see Chapter 5.3).

Concentrations of pregnenolone sulphate and progesterone in individual plasma samples collected from ovarian veins (Chapter 4.1.3) of sows throughout the oestrous cycle and between Days 11 and 15 of pregnancy are
Table 13: Estimated rates of production of steroids by ovaries of unmated gilts between Days 11 and 17 after oestrus ($F = 7053 \, 1/24 \, h$)

<table>
<thead>
<tr>
<th>STEROID</th>
<th>A (nM *)</th>
<th>V (nM +)</th>
<th>PR (umoles/24 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C21</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pregnenolone</td>
<td>3.36</td>
<td>9.90</td>
<td>46</td>
</tr>
<tr>
<td>Pregnenolone sulphate</td>
<td>37.3</td>
<td>56.5</td>
<td>135</td>
</tr>
<tr>
<td>Progesterone</td>
<td>72.7</td>
<td>95.1</td>
<td>158 #</td>
</tr>
<tr>
<td>C19</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DHEA</td>
<td>0.10</td>
<td>0.19</td>
<td>0.63</td>
</tr>
<tr>
<td>Androstenedione</td>
<td>0.45</td>
<td>1.35</td>
<td>6.35</td>
</tr>
<tr>
<td>Testosterone</td>
<td>0.85</td>
<td>0.21</td>
<td>-</td>
</tr>
<tr>
<td>C18</td>
<td>0.03</td>
<td>0.04</td>
<td>0.07</td>
</tr>
</tbody>
</table>

Table 14: Estimated rates of production of steroids by ovaries of pregnant gilts between Days 11 and 17 after mating ($F = 854 \, 1/24 \, h$)

<table>
<thead>
<tr>
<th>STEROID</th>
<th>A (nM *)</th>
<th>V (nM +)</th>
<th>PR (umoles/24 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C21</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pregnenolone</td>
<td>3.88</td>
<td>47.54</td>
<td>37</td>
</tr>
<tr>
<td>Pregnenolone sulphate</td>
<td>25.0</td>
<td>124.8</td>
<td>85</td>
</tr>
<tr>
<td>Progesterone</td>
<td>89.2</td>
<td>409.9</td>
<td>274 #</td>
</tr>
<tr>
<td>C19</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DHEA</td>
<td>0.44</td>
<td>0.60</td>
<td>0.14</td>
</tr>
<tr>
<td>Androstenedione</td>
<td>0.15</td>
<td>8.04</td>
<td>5.90</td>
</tr>
<tr>
<td>Testosterone</td>
<td>0.84</td>
<td>2.97</td>
<td>1.80</td>
</tr>
<tr>
<td>C18</td>
<td>0.03</td>
<td>0.42</td>
<td>0.33</td>
</tr>
</tbody>
</table>

* - 'A', concentration of steroid in ovarian artery, approximated by steroid concentration in external jugular vein (see values in Table 5).

+ - 'V', concentration of steroid in ovarian venous drainage, values from Table 12.

# - As determined by constant infusion (method and derivation of values detailed in Chapter 4.2).
positively correlated \((P<0.001; \text{Text-figure 11})\). While determinants of secretion of pregnenolone sulphate by sow ovaries remain to be elucidated, this correlation implies an association with progesterone secretion.

Pregnenolone, also, may prove to be important during early pregnancy in pigs. This steroid, like its sulphoconjugate, is accumulated in the intra-uterine milieu of pregnant and of non-pregnant gilts (Table 5), and is secreted by porcine ovaries in substantial quantities during the same period (Tables 13 and 14).

Progesterone production between Days 11 and 17 after oestrus is higher in mated animals \((P<0.01; \text{Chapter 4.2})\), and results shown in Tables 13 and 14 indicate that ovarian secretion of testosterone and oestradiol may also be elevated during early pregnancy.

A steroid cocktail administered to maintain pregnancy in ovariectomized sows between Days 11 and 17 of gestation should include those ovarian steroids which cannot be effectively provided from extra-ovarian sources. As a minimum requirement to maintain plasma steroid levels within normal intact ranges, the results of this study indicate that progesterone, pregnenolone sulphate, testosterone and oestradiol should be included in the cocktail; to be administered to sows at rates equivalent to endogenous ovarian secretion (Table 14), as shown below.

**Table 15**: Recommended steroid replacement rates for ovariectomized sows between days 11 and 17 of gestation

<table>
<thead>
<tr>
<th>Steroid</th>
<th>Ovarian secretion/supplementation rate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(umol/24 h)</td>
</tr>
<tr>
<td>C21 Progesterone, Pregnenolone sulphate</td>
<td>274 85</td>
</tr>
<tr>
<td>C19 Testosterone</td>
<td>1.80 0.33</td>
</tr>
<tr>
<td>C18 Oestradiol</td>
<td></td>
</tr>
</tbody>
</table>

# - Administered as the sodium salt.
Text-figure 11: Positive correlation \((P<0.001)\) between levels \((\text{nM})\) of progesterone and of pregnenolone sulphate in individual plasma samples from the ovarian venous drainage of mated (○; between Days 9 and 15 after coitus) and unmated (●; throughout the oestrous cycle) gilts.

\[ y = 28.22 + 1.67x \]
\[ (P<0.001) \]
Where pregnancy has previously been maintained in ovariectomized sows by exogenous steroid treatment (Table 3), only one study has provided steroids at rates which approximate the levels above (viz. Day, Anderson, Emmerson, Hazel & Melampy, 1959). In that study, treatment of ovariectomized sows with 367 umol progesterone/24 h increased embryonic survival (from 26 to 54%), with a further 21% increase in embryonic survival when oestradiol (0.31 umol/24 h) was added to the steroid replacement protocol. No steroid replacement cocktail previously administered to pregnant sows has included testosterone or pregnenolone sulphate.

Those ovarian-derived steroids which can be maintained in peripheral plasma in the absence of ovaries (pregnenolone and androstenedione) may be provided by adrenal secretion (see Heap, Holzbauer & Newport, 1966).

4.4 SUMMARY

By measuring concentrations of steroids in peripheral plasma of intact and oophorectomized gilts, and in ovarian venous plasma of mated and un-mated gilts, the luteal phase sow ovary was shown to be a source of pregnenolone, pregnenolone sulphate, progesterone, DHEA, androstenedione, testosterone and oestradiol. Of the 10 steroids studied, progesterone was shown to be the predominant ovarian secretory product in pregnant and in non-pregnant gilts between Days 11 and 17 after oestrus; endogenous rates of production of this steroid in mated and un-mated animals being near 273 and 158 umoles/24 h respectively (difference significant; \( P<0.05 \)). Estimated rates of release of pregnenolone sulphate by ovaries were also significant (near 100 umoles/24 h), with evidence that ovaries are the principal source of this steroid conjugate in plasma of mated and unmated gilts between Days 11 and 17 after oestrus. Pregnenolone sulphate accumulated in uterine fluid (Table 4) also appears to originate from this plasma pool (Chapter 3.2).

While estimated rates of endogenous production of testosterone and oestradiol (1.80 and 0.33 umol/24 h respectively) were lower than
progestagen release rates, levels of these steroids could not be maintained near physiological levels in the absence of ovaries. Testosterone and oestradiol should thus be included in cocktails formulated to replace endogenous ovarian-steroid secretion in ovariectomized sows between Days 11 and 17 of the oestrous cycle, or of gestation.

4.5 PREGNENOLONE SULPHATE

The experiments detailed in previous Sections have identified pregnenolone sulphate as an abundant component of plasma and of uterine fluid of mated and unmated sows between Days 9 and 15 after oestrus, and have shown this steroid to be a predominant ovarian secretory product. No previous study has reported pregnenolone sulphate in biological compartments of the pig, and there appear to be few reports of its occurrence in any other domestic animal.

Numerous publications have, however, reported the presence of pregnenolone sulphate in human plasma, and the first of these (Eberlein, 1965; Conrad, Pion & Kitchin, 1967; Sjovall, Sjovall & Vihko, 1968) showed that this steroid sulphoconjugate was present at high levels in maternal and in foetal plasma. The insensitive methods of detection used in these early studies (double isotope derivative methods) required large volumes of plasma for assay, so reported values were determined from pooled plasma samples only. Levels of pregnenolone sulphate determined in maternal (e.g. Begue, 1965) and in umbilical cord (Eberlein, 1965) plasma by these investigators were near 120 and 540 nM respectively.

In the late 1960's, more sensitive (to about 100 nM) and precise techniques for detection of pregnenolone sulphate were developed (e.g. solvolysis/gas chromatography; Janne, Vihko, Sjovall & Sjovall, 1969) which enabled quantitation of this steroid in individual patient samples. Using such a technique, Scommegna, Bieniarz & Wineman (1971) measured pregnenolone sulphate levels in human pregnancy plasma, and showed that average plasma
levels at the time of delivery were near 650 nM, about pregnancy values (near 320 nM). Levels of pregnenolone sulphate in fetal plasma were higher than maternal plasma values, with a concentration gradient between the umbilical artery (about 4.06 uM) and umbilical vein (about 2.96 uM) at the time of delivery. These results confirmed earlier evidence that steroid sulphates are not transferred directly from the foeto-placental unit into the maternal circulation (e.g. Diczfalusy, 1965), and that pregnenolone sulphate concentrations in placental blood collected from the intervillous space were not different from peripheral maternal levels (Scommegra, Bienarz & Winemann, 1971). Scommegra et al.'s results also indicated placental clearance of about 1.1 umoles pregnenolone sulphate per litre of blood, in accord with high sulphatase activity in the human placenta (Diczfalusy, 1964), and efficient conversion of pregnenolone sulphate to progesterone in that organ (Pion, Conrad & Wolf, 1966). In pregnancies without a foetal adrenal (e.g. in mothers bearing term anencephalic foetuses), placental progesterone is derived from maternal plasma cholesterol and pregnenolone sulphate (Hellig, Gatterau, Lefebvre & Bolte, 1970). These results justified reappraisal of the early 1960's view that steroid sulphate conjugates were simply metabolic end-products (Calvin & Lieberman, 1966; Gower, 1984) and initiated studies which identified many important metabolic roles for pregnenolone sulphate and other steroid sulphoconjugates.

In their study of pregnenolone sulphate levels in peripheral plasma of humans between birth and adulthood, Peretti and Mappus (1983) measured highest levels in neonates (near 2.6 uM), declining to about 2.1 uM at 24 h, 1.3 uM at one month, 266 nM at between 4 and 6 months, 89 nM at between 7 and 12 months, approaching a nadir of about 41 nM between 2 and 9 years of age. A progressive rise was then shown until adulthood, values in healthy adults being near 130 nM. Plasma pregnenolone sulphate levels are similar in both sexes (Peretti & Mappus, 1983).

Pregnenolone sulphate in plasma appears to originate from the adrenal in
neonates (Little, Billiar, Rahman, Johnson, Takaoka & White, 1975) and in adults (Wieland, DeCourcy, Levy, Zala & Hirschmann, 1965; Fukushima, Bradlow, Hellman & Gallagher, 1963; Arcos, Gurpide, Van de Wiele & Lieberman, 1964; Conrad, Pion & Kitchin, 1967; Buster, Chang, Preston, Blashoff, Cousins, Abraham, Hobel & Marshall, 1979). These changes in plasma pregnenolone sulphate levels with age reflect changes in the pattern of secretion of this steroid by the foetal zone of the adrenal cortex, and involution of that secretory tissue (Peretti & Forrest, 1978). Pregnenolone sulphate does not appear to be involved in secretory changes of the adrenarche (i.e. does not rise as abruptly as does DHEA sulphate at about age 7 yrs.; ibid). During normal pregnancy, levels of pregnenolone sulphate in plasma approximate those of progesterone (Begue, 1965).

Quantitatively, pregnenolone sulphate is thus a significant steroid in the human, endogenous rates of production in adult men and women being near 34 umoles/day (Wang, Bullbrook, Ellis & Coombs, 1967) with a metabolic clearance rate about 10 times higher than that of its precursors (e.g. cholesterol sulphate) or products (e.g. DHEA sulphate; Wang et al., 1967). However, pregnenolone sulphate is not, itself, progestational (Deghenghi & Revesz, 1965) and there has been much speculation regarding physiological roles of this and other steroid sulphates in mammals. Studies described above have demonstrated the importance of foetal pregnenolone sulphate as a precursor to placental progesterone synthesis and, subsequently, pregnenolone sulphate was shown to be an important precursor to corticosteroidogenesis (e.g. Dominguez, Valencia & Laza, 1975). In this regard, adrenocorticotropic hormone (ACTH) increases pregnenolone concentrations by stimulating sulphatase activity (ibid), the more effective desulphation of pregnenolone sulphate (compared to DHEA sulphate) in this study being in accord with its high metabolic clearance rate (Wang et al., 1967). Diurnal changes in plasma levels of pregnenolone sulphate and of cortisol are also parallel, as are depressions in plasma levels of these steroids following dexamethasone treatment (Peretti & Mappus, 1983). These
same investigators found that rises in plasma levels of pregnenolone sulphate and ACTH following withdrawal of 11-\(\beta\) hydroxylase inhibitors (e.g. metapirone ditartate) were closely correlated. These results indicate that the measurement of pregnenolone sulphate in plasma may provide a useful index of ACTH production.

None of the enzymes involved in steroid hormone biosynthesis after pregnenolone seem to limit the quantitative production of the final corticoids, and these enzymes are not stimulated by ACTH (Koritz & Kumar, 1970). There is increasing evidence that steroid sulphates may control the activity of certain steroid transforming enzymes (Makin, 1984) and influence the transport of steroids in blood (Vihko, 1966). More significantly, steroid sulphates provide a pool of circulating steroid precursors. In this respect, the 3-sulphate group must be cleaved before any 5-en-3-\(\beta\)-ol steroid sulphate can be transformed into its corresponding 4-ene-3-keto steroid. The sequence of conversions from cholesterol to DHEA via pregnenolone and 17-\(\alpha\)-hydroxypregnenolone occurs for the free 5-ene-3-\(\beta\)-hydroxy-\(\alpha\)-ol steroids and for their sulphates, in which case the sulphate group remains within the molecule throughout the conversion. The participation and regulation of steroid sulphatase activity, and roles for steroid sulphates as precursors in the biosynthesis of active steroid hormones, have been reported for adrenal, placental and gonadal tissues (see Dominguez, Valencia & Loza, 1975). Thus, while it is known that levels of pregnenolone sulphate in plasma of women during the menstrual cycle fluctuate between maximum levels during the luteal phase of the cycle (about 250 nM) and minimum values at about the time of ovulation (about 140 nM; Aso, Aedo & Cekan, 1977), it is not known whether the ovary contributes to these levels directly (as it does in the pig; Chapters 4.1 and 4.3), indirectly (e.g. by affecting adrenal secretion), or not at all.

In these and other studies which have reported the occurrence and metabolism of pregnenolone sulphate, this steroid conjugate has been ascribed many potentially significant roles in regard to placental and
adrenal steroidogenesis, steroid transport, and control of enzyme activity.

The pool of pregnenolone sulphate in uterine fluids of gilts between Days 9 and 15 after mating thus represents a potentially important source of steroid precursor, but there is currently no evidence that preimplantation blastocysts can metabolize this $C_{21}$ steroid sulphoconjugate. The following experiments have been designed to make good this deficiency.
CHAPTER V

METABOLISM OF STEROID HORMONES BY CULTURED PIG BLASTOCYSTS

5.1 INTRODUCTION

Experiments detailed in the previous chapters have reported high concentrations of pregnenolone sulphate in uterine washings of early pregnant sows, and have investigated origins of this and other steroids which are accumulated in the uterine lumen during the preimplantation phase. On the basis of these results, it was postulated that steroid sulphoconjugates concentrated in histotroph could serve as a precursor pool for steroidogenesis by preimplantation blastocyst tissues in vivo. The following experiments examine aspects of metabolism of pregnenolone sulphate by intact pig blastocysts in vitro.

As existing culture techniques are ineffective in maintaining a majority of pig embryos beyond hatching (e.g. Kuzan & Wright, 1982), and the developmental phase of particular interest in this Thesis is initiated at hatching, a preliminary study investigates culture requirements for blastocysts between Days 3 and 9 after fertilization.
5.2 COLLECTION OF PIG EMBRYOS AND IMPROVEMENT OF A TECHNIQUE FOR THEIR CULTURE BEYOND HATCHING IN VITRO

5.2.1 Introduction

There are now several reports of successful culture of 4-8 cell pig embryos to the blastocyst stage in vitro, but attempts to maintain embryos beyond this stage have had limited success. Davis & Day (1978) reported partial hatching of 7/30 pig blastocysts following culture from the 4-cell stage and Lindner & Wright (1978) observed complete hatching in only 3/35 expanded blastocysts. In their study of the effects of serum on development of cultured pig blastocysts, Robl & Davis (1981) reported complete hatching in 4/22 pig embryos and, more recently, Kuzan & Wright (1982) reported 9/21 embryos successfully hatched when co-cultured with bovine fibroblasts.

Shaffer & Wright (1978) suggested similar requirements for pig and mouse embryos in culture, and the conditions chosen in various pig embryo studies have thus been based on those recommended for mouse embryo culture. However, the failure to consistently obtain hatching and development of embryos beyond the blastocyst stage in the pig indicates differing culture requirements at the later developmental stages. In this respect, published evidence suggests that energy substrate requirements of pig embryos differ from other mammalian embryos. For example, pyruvate, at concentrations (0.25 mM) which support blastocyst development in the mouse and rabbit (Brinster, 1965 a, c; 1969), seems to inhibit in vitro embryonic development in the pig (Davis & Day, 1978). Furthermore, while some media for culture of pig embryos contain porcine serum, Hermann, Schafer & Holtz (1981) and Robl & Davis (1981) have reported improved development of pig blastocysts cultured in media supplemented with various heterologous proteins.

The present study was designed to examine more closely the energy and protein requirements of preimplantation pig embryos, with particular reference to establishing reliable methods for culturing embryos beyond
hatching from the zona pellucida.

5.2.2 Materials and methods

Flush/transport medium

The flush/transport medium used in the collection and transport of embryos was prepared from powdered Minimal Essential Medium (MEM, Eagle, with Earle's salts, with L-glutamine; GIBCO, New York, N.Y., U.S.A.) dissolved in 6x glass distilled rain water and supplemented with 21 mM Hepes, 4 mM sodium bicarbonate and 5 mg/ml bovine serum albumin (BSA; Fraction V, Sigma, St. Louis). Antibiotic/antimycotic solution (GIBCO) was added to provide 100 i.u. Penicillin, 100 ug Streptomycin and 25 ug amphotericin B per ml of final solution. The pH and osmolarity of the medium were adjusted to published optima for mammalian embryos (7.40 and 280 mOs respectively; Brinster, 1972). The prepared medium was sterilized by filtration (Millex-GS 0.22 μM filter, Millipore Corporation, Bedford, Massachusetts, U.S.A.) and stored at 4°C.

Embryo collection

Large White sows were injected intramuscularly (i.m.) with 800 i.u. Pregnant Mare's Serum Gonadotrophin (PMSG, Folligon, Intervet Australia Pty. Ltd., Victoria, Australia) on the day of weaning, then mated on each day of standing-heat. Sows not mated within one week of PMSG treatment were excluded from the experiment. Animals were slaughtered by electrical stunning and exsanguination 3-4 days following the first mating, and the uteri were removed from the carcases within six minutes of stunning. Fifteen ml of warm (37°C) flush/transport medium was flushed from the fimbrial end of each fallopian tube to a point in the uterine body, 20 cm distal to the uterotubal junction (see Stone et al., 1984 a). Flushings were
collected directly into sterile, conical base containers (30 ml Sterile Universal container, Filtrona, Thomastown, Victoria, Australia).

Culture media

Experiment 1: Energy sources. Two basal culture media were used. The first, Krebs-Ringer Bicarbonate (KRB), was prepared according to Brinster (1965 a; RMOC-2), with the exclusion of sodium lactate and sodium pyruvate and inclusion of glucose (1 mg/ml) and BSA (5 mg/ml). The second medium (MEM) was the same as the flush/transport medium described above, with sodium bicarbonate substituted for HEPES on an equimolar basis. This medium contained glucose (1 mg/ml). Media containing combinations of the energy sources; glucose (G), lactate (L, 25.0 mM; DL-Lactic acid, sodium salt, SIGMA, St. Louis, Missouri, U.S.A.) and pyruvate (P, 0.25 mM; Pyruvic acid, sodium salt, SIGMA) were prepared; viz. G+L+P, G+L, G+P and G only. Osmolarity was maintained near calculated isotonicity by adjusting the NaCl content of each medium. Following gassing (5% CO₂, 5% O₂, 90% N₂), the pH of each medium was adjusted to 7.40 and osmolarity was finally adjusted to 280 mOs with water if required. Media were filter sterilized (0.22 µm) and handled aseptically thereafter.

Experiment 2: Protein sources. Aliquots of MEM with glucose (as detailed above, but without BSA) were supplemented with one of four protein sources; BSA (5 mg/ml), heat-inactivated (56°C for 30 min) pig serum (HIPS, 10% v/v), heat-inactivated foetal calf serum (HIFCS, 10% v/v; CSL, Victoria, Australia) or heat-inactivated human serum (HIHS, 10% v/v). The osmolarity and pH of the prepared media were adjusted to 280 mOs and 7.40 respectively, and the media sterilized, as described above.
Culture

Embryos recovered from uterine flushings from each animal were washed twice in flush/transport medium, transported to the culture laboratory (1 h at about 35°C) and there divided into equivalent subgroups (8 in Experiment 1; 4 in Experiment 2). Embryos within each sub-group were washed twice in the protein supplemented medium in which those embryos were then to be cultured. Washed embryos were transferred to 50-μL droplets of medium which had been equilibrated at 37°C under sterile paraffin oil, saturated with 5% CO₂ : 5% O₂ : 90% N₂, in fully vented petri dishes (Kayline Plastics, Thebarton, South Australia). During culture, dishes were maintained in a humidified gas environment of 5% CO₂ : 5% O₂ : 90% N₂, and at 37°C (Precision CO₂ Incubator, Contherm Scientific Company, Petone, New Zealand). Embryos were examined daily at 40x magnification during the ensuing six days of culture. The morphological characteristics by which embryos were classified were: formation of a blastocoele cavity, fracture of the zona pellucida, complete hatching of the blastocyst from the zona, and expansion of the hatched blastocyst to a diameter exceeding that of the discarded zona. Cultured embryos were not stained to determine numbers of nuclei. Media were not changed during the culture period.

Statistics

Between-treatment differences in proportions of cultured embryos reaching particular stages of development in each experiment were compared by Chi-square analysis (Clarke, 1969).

5.2.3 Results

Results of Experiment 1 are summarized in Tables 16 and 17. Overall, more
Table 16: In vitro development of 4-8 cell pig embryos cultured for six days in KRB and MEM based media

<table>
<thead>
<tr>
<th>Culture medium*</th>
<th>Number of embryos cultured</th>
<th>Proportion (%) of embryos which developed to:</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Blastocyst</td>
<td>Hatching</td>
</tr>
<tr>
<td>KRB</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G + L + P</td>
<td>20</td>
<td>45.0 a¹</td>
<td>0 b</td>
</tr>
<tr>
<td>G + L</td>
<td>20</td>
<td>65.0 a</td>
<td>5.0 be</td>
</tr>
<tr>
<td>G + P</td>
<td>18</td>
<td>50.0 a</td>
<td>0 b</td>
</tr>
<tr>
<td>G alone</td>
<td>22</td>
<td>68.2 a</td>
<td>9.1 bde</td>
</tr>
<tr>
<td>Over all energy² supplements</td>
<td>80</td>
<td>57.5 f</td>
<td>3.8 f</td>
</tr>
<tr>
<td>MEM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G + L + P</td>
<td>18</td>
<td>55.6 a</td>
<td>33.3 acd</td>
</tr>
<tr>
<td>G + L</td>
<td>19</td>
<td>63.2 a</td>
<td>26.3 acde</td>
</tr>
<tr>
<td>G + P</td>
<td>19</td>
<td>68.4 a</td>
<td>21.1 acde</td>
</tr>
<tr>
<td>G alone</td>
<td>21</td>
<td>71.4 a</td>
<td>38.1 ac</td>
</tr>
<tr>
<td>Over all energy supplements</td>
<td>77</td>
<td>64.9 f</td>
<td>29.9 g</td>
</tr>
</tbody>
</table>

*G, 5.56 mM glucose; L, 25 mM sodium lactate; P, 0.25 mM sodium pyruvate.

¹ Within a column, values following by the same letter do not differ significantly (P < 0.05).

² Over all energy supplements, mean values for KRB and MEM followed by the same letter do not differ significantly (P < 0.05).
Table 17: In vitro development of 4-8 cell pig embryos cultured for six days in different combinations of energy sources (KRB and MEM data pooled)

<table>
<thead>
<tr>
<th>Energy* source</th>
<th>Number of embryos cultured</th>
<th>Proportion (%) of embryos which developed to:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Blastocyst</td>
</tr>
<tr>
<td>G + L + P</td>
<td>38</td>
<td>50.0 a¹</td>
</tr>
<tr>
<td>G + L</td>
<td>39</td>
<td>64.1 a</td>
</tr>
<tr>
<td>G + P</td>
<td>37</td>
<td>59.5 a</td>
</tr>
<tr>
<td>G alone</td>
<td>43</td>
<td>69.8 a</td>
</tr>
</tbody>
</table>

* G, L, P defined beneath Table 16

¹ Within a column, values followed by the same letter do not differ significantly (p at 0.05)
embryos commenced hatching, hatched and expanded (diameter of blastocyst > diameter of discarded zona pellucida) in MEM based media than KRB based media (Table 16, \( P < 0.05 \)). When MEM and KRB data were pooled (Table 17), in \textit{vitro} development beyond hatching was greatest in G-only supplemented media, although the differences between G alone and G+L media were not significant (\( P > 0.05 \)).

Statistical comparison of data from individual media showed MEM+G alone to be superior to all other media (\( P < 0.05 \)) in its capacity to support development of 4-8 cell pig embryos to hatched/expanded blastocyst stages (Table 16). MEM+G was thus used as the basal medium (with the exclusion of BSA) in Experiment 2, the results of which are summarized in Table 18. As a supplement to MEM+G, HIPS was inferior to BSA, HIFCS and HIHS (\( P < 0.05 \); Table 18). The medium supplemented with HIHS was superior to all others in maintenance of pig embryos to the hatched/expanded blastocyst stages (\( P < 0.05 \); Table 18).

The uniformity of procedures between Experiments 1 and 2 is reflected in the similarity of results of culture of 4-8 cell embryos in MEM+G+BSA in each Experiment (Tables 16 and 18).

5.2.4 Discussion

Davis & Day (1978) reported improved pig blastocyst formation in synthetic media without pyruvate. The present results show inhibition of blastocyst development in media supplemented with 0.25 mM pyruvate (\( P < 0.05 \); Table 17), but inhibitory effects of pyruvate on blastocyst formation were not significant. Many factors may contribute to this discrepancy between results of the two studies, including differences in genotypes of embryos and differences in culture conditions. Regardless, there is no evidence in either study to suggest a requirement for 0.25 mM pyruvate in pig blastocyst formation or development in \textit{vitro}. Furthermore, this study has shown an inhibitory effect of 25 mM lactate on blastocyst development beyond the
Table 18: *In vitro* development of 4-8 cell pig embryos cultured for six days in MEM supplemented with different protein sources

<table>
<thead>
<tr>
<th>Protein* supplement</th>
<th>Number of embryos cultured</th>
<th>Proportion (%) of embryos which developed to:</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Blastocyst</td>
<td>Hatching</td>
</tr>
<tr>
<td>BSA</td>
<td>39</td>
<td>87.2 a&lt;sup&gt;1&lt;/sup&gt;</td>
<td>46.2 b</td>
</tr>
<tr>
<td>HIPS</td>
<td>34</td>
<td>55.9 b</td>
<td>2.9 c</td>
</tr>
<tr>
<td>HIFCS</td>
<td>35</td>
<td>80.0 a</td>
<td>60.0 ab</td>
</tr>
<tr>
<td>HIHS</td>
<td>39</td>
<td>84.6 a</td>
<td>79.5 a</td>
</tr>
</tbody>
</table>

*BSA, 5 mg bovine serum albumin/ml; HIPS, heat-inactivated (56°C for 30 min) pig serum (10% v/v); HIFCS, heat-inactivated foetal calf serum (CSL, Vic., Australia) (10% v/v); HIHS, heat-inactivated human serum (10% v/v).

<sup>1</sup> Within a column, values followed by the same letter do not differ significantly (P < 0.05).
commencement of hatching, and the data suggest that the pyruvate and lactate effects are additive (Table 17). Pyruvate has previously been shown to be an important energy source for most mammalian embryos in early stages of development but, as discovered by Whitten (1970) when optimizing culture conditions for mouse embryos, there is a critical range of concentrations of pyruvate which will support formation of blastocysts. It would thus seem more likely that the reported inhibitory effect of pyruvate relates to a detrimental effect of excess substrate rather than the energy sources of pig embryos differing from the general pattern for other mammalian embryos (Brinster & Troike, 1979). Further studies to optimize the levels of pyruvate and lactate consistent with maintenance of the appropriate redox potential within pig embryos will be required to clarify this situation.

A protein source has been shown to be necessary for in vitro development of blastocysts from laboratory animals (e.g. Gwatkin & Meckley, 1965). If culture media are supplemented with a non-polymerizable polymer such as Ficoll or polyvinylpyrrolidone, development of pig embryos in vitro is arrested (Davis & Day, 1978; Herrmann et al., 1981). Developmental responses to supplementary protein have been attributed to effects of amino acids contributed by the protein source (Brinster, 1972), including the provision of those amino acids which may be essential to blastocyst expansion in vitro (Daniel & Krishnan, 1967), stabilization of the blastocyst membranes by the protein (Brinster, 1965 b), chelation of traces of toxic metal ions (in particular copper and zinc) by the protein amino groups (Whitten, 1970), and energy provision via protein-bound fatty acids (Kane & Headon, 1980). Several studies have examined effects of different protein sources on development of pig morulae and blastocysts in vitro, the most effective supplement to date being 10% (v/v) sheep serum or FCS (Robl & Davis, 1981). Pig serum seems to support cleavage of 2-cell pig embryos (Herrmann et al., 1981) but is less effective in stimulating post-blastocyst development in vitro (Robl & Davis, 1981). The results reported here are consistent with the conclusions of Robl & Davis (1981) in that MEM
supplemented with BSA (5 mg/ml) or FCS (10% v/v) supported both blastocyst formation and post-blastocyst development of cultured pig embryos better ($P<0.05$) than did HIPS (10% v/v; Table 18). However, HIHS (10% v/v) proved superior to both HIPS and BSA ($P<0.05$) in its capacity to support blastocyst formation and hatching, and stimulated expansion of hatched blastocysts better ($P<0.05$) than did HIPS, BSA or HIFCS (Table 18). Over 50% (22/39) of 4-8 cell pig embryos cultured in MEM supplemented with 10% (v/v) HIHS developed to hatched and expanded blastocysts (Table 18). This proportion is higher than any other reported for in vitro culture of pig embryos during the same developmental period and HIHS is thus recommended as a protein supplement to MEM based media for pig embryo culture.
5.3 RELEASE OF STEROIDS BY CULTURED PIG BLASTOCYSTS: THE EFFECT OF SUPPLEMENTING THE CULTURE MEDIUM WITH PREGNENOLONE SULPHATE OR WITH AN INHIBITOR OF 3-β HYDROXysteroid dehydrogenase

5.3.1 Introduction

With the exception of the side-chain cleavage enzyme required for conversion of cholesterol to pregnenolone, all enzymes necessary for the synthesis of oestrogens from acetate have been identified in trophectoderm tissues of pig embryos (see Chapter 2.5.3.1), and the capacity for dispersed blastocyst tissue to synthesize oestrogens (oestrone and oestradiol) from C₂₁ and C₁₉ steroids in vitro has been demonstrated (Gadsby et al., 1976; Heap et al., 1981). However, pig blastocysts cannot synthesize pregnenolone from acetate or cholesterol (Flint et al., 1979), so the nature of the dependence of porcine blastocysts on maternal C₂₁ steroids as precursors for de novo steroid synthesis is uncertain.

In view of circumstantial evidence for sulphatase activity in pig blastocysts (Perry et al., 1976; Flint et al., 1979), and evidence for concentration of pregnenolone sulphate in uterine fluid of early pregnant sows (Tables 4 and 5), this C₂₁ steroid conjugate may be an important precursor to oestrogen synthesis by pig blastocysts.

The present study examines this hypothesis by measuring the rate of secretion of steroids by pig blastocysts cultured in media supplemented with pregnenolone sulphate. To ascertain whether this release reflects steroid synthesis or dilution of steroids concentrated within the blastocoel cavity, comparison is made with steroid secretion by blastocysts cultured in media supplemented with an inhibitor of 3-β hydroxysteroid dehydrogenase (HSD) activity.
5.3.2 Materials and methods

General

Collection of embryos. Large White sows were injected i.m. with 800 i.u. PMSG (Polligon, Intervet Australia Pty. Ltd., Victoria, Australia) on the day of weaning, then mated on each day of standing heat. Embryos were recovered from uteri at slaughter, following the protocol detailed previously (Stone et al., 1984 a).

Basic culture medium and culture conditions. Culture media were prepared from powdered MEM, supplemented with 25 mM sodium bicarbonate and 10% (v/v) human serum, as detailed in Chapter 5.2.

Embryos were cultured in glass, stainless-steel capped, culture tubes in a humidified gas environment (5% CO₂, 5% O₂, 90% N₂) at 37°C (Precision CO₂ Incubator, Contherm Scientific Company, Petone, New Zealand). At the termination of culture, embryos were classified according to morphological characteristics (Stone, Quinn & Seamark, 1984; Chapter 5.2.2).

Experiment 1: Release of pregnenolone and progesterone by blastocysts cultured in different concentrations of pregnenolone sulphate

Pregnenolone sulphate (sodium salt; Sigma Chemical Co., St. Louis, Missouri, USA) was added to aliquots of the basic culture medium to provide final concentrations of pregnenolone sulphate (as determined by radioimmunoassay; Stone & Seamark, 1985) of 10.4, 12.8, 17.0 and 4470 nM.

Eleven embryos (collected five days after mating; unhatched blastocyst stage) assigned to each of the four treatment media were washed twice in the medium in which the embryos were then to be cultured, and transferred into
500 μl of the medium in a glass culture tube. Control culture tubes containing each medium, but without blastocysts, were incubated alongside blastocyst culture tubes.

Following six days culture, treatment and control tubes were centrifuged at low speed (56 g, 20 min, 4°C) and supernatants were recovered and stored at -15°C.

**Experiment 2**: Levels of pregnenolone and progesterone in blastocoel fluid

Three to 11 blastocysts were recovered from saline (0.9%, w/v) washes of uteri of each of 11 sows which had been mated 4 to 8 days previously. Following washing in 0.9% (w/v) saline and measurement of diameters (using an ocular graticule), pooled blastocysts from individual sows were transferred into 100 μl glass-distilled water, frozen (-26°C)/thawed 6x to effect disintegration, and stored at -15°C.

**Experiment 3**: Effect of inhibition of 3β-HSD on release of pregnenolone, progesterone, androstenedione, testosterone, oestrone and oestradiol by pig blastocysts cultured in a medium containing 1 μM pregnenolone sulphate

The control medium (C) was prepared as detailed above, and supplemented with pregnenolone sulphate to a final concentration of 1.0 μM. The treatment medium (T) was prepared similarly, except for the addition of 1.2% (v/v) of a solution (7.8 mM) of Trilostane ([2-α, 4-α, 5-α, 17-β]-4,5-epoxy-17-hydroxy-3-oxoandrostane-2-carbonitrile; Sterling-Winthrop, WIN 24,540) in ethanol. Trilostane is a potent inhibitor of 3β-HSD (Potts, Creange, Harding & Schane, 1978). An equivalent volume of ethanol was added to the control culture medium (to a final level of 1.2%, v/v).

Fifty-two embryos (29 at Day 3 post-fertilization and 23 at Day 6 post-fertilization) recovered from five sows were allocated to five replicate
pairs (three at Day 3; two at Day 6) of culture tubes, with blastocysts in C and T tubes of each pair being littermates. Blastocysts were divided between C and T tubes within each pair as evenly as was possible, and were cultured for four days. At termination of culture, culture tubes were centrifuged (56 g, 20 min, 4°C) and the supernatants aspirated and stored at -15°C.

Recovery and separation of steroids following culture or lysis

In Experiments 1 and 2, retained media were thawed and aliquots extracted with 2.5 ml hexane:ethyl acetate (9:1; v/v). Extracts were concentrated and fractionated to separate progesterone and pregnenolone, as detailed previously (Chapter 3.2.2.2).

Aliquots of supernatants from Experiment 3 were extracted with 2.5 ml hexane:ethyl acetate (9:1; v/v) for recovery of progesterone and pregnenolone (as above) or with heptane:ethyl acetate (2:3; v/v) for recovery of androstenedione, testosterone, oestrone and oestradiol. The latter four steroids were separated by Lipidex chromatography (Chapter 3.2.2.2).

Steroid assays

Levels of pregnenolone, progesterone, androstenedione, testosterone, oestrone and oestradiol in chromatography eluents, and of pregnenolone sulphate in culture media, were determined using validated radioimmunoassays, as detailed elsewhere (Stone & Seamark, 1985; see Chapter 3.2.2.2).
Calculation of rate of release of steroids by cultured blastocysts

Concentrations of each steroid measured were corrected for blank values and the change in total net content of each steroid in each culture tube was determined. These values were then divided by the number of blastocysts and by the number of days of culture appropriate to each tube. The resultant average value (pmoles/blastocyst/day) provided the index of steroid release.

Determination of progesterone and pregnenolone content of blastocysts

Following blank (distilled water) correction of concentrations of pregnenolone and progesterone in aliquots of the lysis medium, the total net content of the steroids in each tube, and in each blastocyst, were calculated. For each group of embryos, the total blastocyst volume was determined, and the concentration of pregnenolone and progesterone in that volume calculated.

5.3.3 Results

Rates of release of pregnenolone and progesterone from blastocysts (pmol/day) cultured in different concentrations of pregnenolone sulphate are summarized in Text-figure 12. This data shows that pig blastocysts recovered from gilts five days after mating can release pregnenolone at a rate near 0.6 pmoles/day when cultured in media supplemented with pregnenolone sulphate to between 10.4 and 17.0 nM. The rate of release of pregnenolone increased in parallel to increasing concentrations of pregnenolone sulphate in the culture medium throughout this range, rising further to 1.7 pmoles/day for blastocysts cultured in a medium supplemented with pregnenolone sulphate to 4.47 uM. The results summarized in Text-figure 12 also show release of progesterone by pig blastocysts in vitro, with rising rates of progesterone
Text-figure 12: Release of progesterone (O) and pregnenolone (●) by pig blastocysts cultured from Day 5 after fertilization (Experiment 1), in the presence of different concentrations of pregnenolone sulphate (pmol/blastocyst/day)
secretion (0.07 to 0.38 pmol/blastocyst/day) in parallel with increasing pregnenolone sulphate concentrations in the culture medium.

Concentrations of pregnenolone and progesterone in blastocoele fluid of freshly collected blastocysts are shown in Table 19. At the termination of culture in Experiment 1, between 5 and 7 of the 11 embryos in each treatment were hatching, or had hatched from the zona pellucida.

Results of Experiment 3, showing effects of Trilostane on release of steroids, are presented in Table 20.

The determinations for steroid release by blastocysts are average values, and blastocyst steroid contents are average values calculated from measurement of blastocyst dimensions and the total amount of steroid measured in the lysis medium. The steroid release/content data are thus not amenable to further statistical analysis, and are summarized as means (± s.e.m.) derived from replicate determinations within each treatment.

5.3.4 Discussion

Enhanced pregnenolone release by blastocysts cultured in the presence of higher concentrations of pregnenolone sulphate (Text-figure 12) is consistent with previous reports of high aryl sulphatase activity in dispersed blastocyst tissues of the pig (Perry et al., 1976; Flint et al., 1979). Furthermore, in view of high concentrations of pregnenolone sulphate in uterine flushings of sows during early pregnancy (about 4 µM between Days 9 and 15 after mating; Table 5), secretion of pregnenolone by conceptus tissue in utero could exceed 1.7 pmol/blastocyst/day.

Dickmann, Dey & Sen-Gupta (1976) detected 3-β HSD activity in early pig embryos (8-cell, about Day 3 post fertilization), and the present evidence for progesterone secretion by cultured blastocysts (Text-figure 12) indicates 3-β HSD activity in intact pig embryos between Days 5 and 9 after fertilization. Depressed rates of secretion of progesterone by blastocysts cultured in media containing a 3-β HSD inhibitor (Table 20)
Table 19: Pregnenolone and progesterone content of pig blastocysts (mean ± s.e.m.)

<table>
<thead>
<tr>
<th></th>
<th>Day 4-5 after coitus (Unhatched)</th>
<th>Day 6-8 after coitus (Hatched)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of sows</td>
<td>4</td>
<td>7</td>
</tr>
<tr>
<td>Total number of blastocysts</td>
<td>28</td>
<td>49</td>
</tr>
<tr>
<td>Blastocyst volume (nL)</td>
<td>1.13 ± 0.09</td>
<td>5.3 ± 1.4</td>
</tr>
<tr>
<td>Steroid content per blastocyst (fmol)</td>
<td>5.4 ± 2.5</td>
<td>20.8 ± 8.9</td>
</tr>
<tr>
<td>Concentration in blastocoel fluid (μM)</td>
<td>4.8 ± 2.4</td>
<td>22.2 ± 9.2</td>
</tr>
<tr>
<td>Concentration in free uterine fluid (μM)*</td>
<td>0.039</td>
<td>0.017</td>
</tr>
</tbody>
</table>

* from Table 6; Stone & Seamark, 1985.
Table 20: Release of steroids by cultured pig blastocysts (fmol/day; mean ± s.e.m.), and the effect of supplementation of the culture medium with Trilostane.

<table>
<thead>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total number of blastocysts</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>15</td>
<td>11</td>
<td>26</td>
<td></td>
</tr>
<tr>
<td>T</td>
<td>14</td>
<td>12</td>
<td>26</td>
<td></td>
</tr>
<tr>
<td>Number of replicates</td>
<td>C&amp;T</td>
<td>3</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>Steroid</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>P</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Steroid</td>
<td>Rate of release of steroid (fmol/blastocyst/day)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C&lt;sub&gt;21&lt;/sub&gt;</td>
<td>Pregnenolone</td>
<td>C</td>
<td>98.8 ± 82.3</td>
<td>610 ± 518</td>
</tr>
<tr>
<td></td>
<td></td>
<td>T</td>
<td>60.0 ± 46.7</td>
<td>1691 ± 1311</td>
</tr>
<tr>
<td></td>
<td>Progesterone</td>
<td>C</td>
<td>13.3 ± 6.5</td>
<td>87.0 ± 49.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>T</td>
<td>0.0 ± 0.0</td>
<td>2.8 ± 0.0</td>
</tr>
<tr>
<td>C&lt;sub&gt;19&lt;/sub&gt;</td>
<td>Androstenedione</td>
<td>C</td>
<td>1.3 ± 0.7</td>
<td>60.4 ± 42.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>T</td>
<td>5.1 ± 4.9</td>
<td>6.8 ± 6.8</td>
</tr>
<tr>
<td></td>
<td>Testosterone</td>
<td>C</td>
<td>16.9 ± 8.1</td>
<td>40.2 ± 18.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>T</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>C&lt;sub&gt;18&lt;/sub&gt;</td>
<td>Oestrone</td>
<td>C</td>
<td>0.4 ± 0.1</td>
<td>1.2 ± 0.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>T</td>
<td>1.8 ± 0.7</td>
<td>1.9 ± 1.3</td>
</tr>
<tr>
<td></td>
<td>Oestradiol</td>
<td>C</td>
<td>6.6 ± 2.2</td>
<td>12.3 ± 6.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>T</td>
<td>11.4 ± 7.3</td>
<td>7.3 ± 3.5</td>
</tr>
<tr>
<td>Number of embryos hatching, or hatched, from the zona pellucida at the time of termination of culture</td>
<td>C</td>
<td>3</td>
<td>11</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>5</td>
<td>11</td>
<td>16</td>
</tr>
</tbody>
</table>

* Treatment;
  C, Control
  T, Trilostane (Sterling-Winthrop, WIN 24,540).
support this conclusion. Possible pathways of pregnenolone sulphate metabolism by pig blastocysts are indicated in Text-figure 13.

In regard to the biological significance of progesterone secretion by preimplantation blastocysts in vivo, this C21 steroid is known to exert local effects upon the endometrium, including the stimulation of release of protein (Knight, Bazer & Wallace, 1973; Schlosnagle et al., 1974; Adams, Bazer & Roberts, 1981), and activation of peptidase and lysozyme activity in endometrial tissues (Roberts et al., 1976). Progesterone also induces an inhibitor of plasmin/trypsin activity which appears in uterine secretions of early pregnant pigs, and which may prevent degradation of those macro-molecules which are essential to conceptus development (Fazleabas, Bazer & Roberts, 1982). Furthermore, progesterone is a potentially important immuno-suppressive steroid (Siiteri et al., 1977; Fujisaki, Kawano, Haruyama & Mori, 1985) so its secretion by preimplantation pig blastocysts, and concentration at the trophectoderm boundary layer, could effect immunologic tolerance of embryonic tissues by the sow. In respect to these potential roles for progesterone in early pregnancy in the pig, secretion of progesterone by pig blastocysts may prove essential to preimplantation embryonic growth and survival in vivo, and diminished levels of progesterone precursors in histotroph and/or impaired sulphatase/3-β-HSD activity in blastocyst tissues may account for a proportion of the high level of embryonic loss which occurs immediately prior to implantation in this species (Scofield et al., 1974).

The average total content of pregnenolone and progesterone appeared higher in blastocysts collected from sows at six days after mating, in comparison with Day 4-5, although there was no evidence for change in the concentrations of either steroid in blastocoel fluid during blastocyst expansion (see Tables 19 and 5). Progesterone levels in blastocysts were higher than those of pregnenolone, and average concentrations of both steroids in blastocoel fluid exceeded concentrations in uterine fluid by more than 200 fold (Table 19). While this seems to be the first documented
Text-figure 13 : Pathways for sex steroid biosynthesis from cholesterol

Index to participating enzymes:

1. Sterol side-chain cleavage enzyme (complex)
2. 17α hydroxylase; C-17,20 lyase
3. 3β hydroxysteroid dehydrogenase (3β HSD)
4. Sulphotransferase
5. Aryl sulphatase
7. 17β oxidoreductase
8. 17β hydroxysteroid dehydrogenase (17β HSD)
9. Aromatase
Cholesterol → Pregnenolone sulphate → DHEA sulphate → Pregnenolone → Progesterone → Androstenedione → Testosterone → Oestradiol-17β → Oestrone sulphotase → Oestrone → Oestrone sulphate
evidence for concentration of pregnenolone and progesterone in intact preimplantation pig blastocysts, accumulation of progesterone and progesterone metabolites in preimplantation rabbit embryos has been reported previously (Seamark & Lutwak-Mann, 1972; Borland, Erickson & Ducibella, 1977).

Cultured pig blastocysts also secreted significant amounts of testosterone (Table 20), rates of secretion of this steroid being diminished in the presence of the 3-β HSD inhibitor (Table 20). DHEA or pregnenolone may thus act as precursors to testosterone synthesis by pig blastocysts in vitro. Effects of Trilostane on secretion of androstenedione, oestrone and oestradiol were less marked (see Table 20).

Enhanced pregnenolone 'secretion' in the presence of Trilostane (Table 20) may reflect lower rates of metabolism of pregnenolone when 3-β HSD activity is inhibited. This effect was not investigated further.

While the predominant secretory products of control blastocysts in Experiment 3 (collected six days after mating) were pregnenolone and progesterone (Table 20), their respective rates of release (about 0.6 and 0.09 pmol/day respectively; Table 20) were lower than would have been predicted from the results of Experiment 1 (about 1.0 and 0.20 pmol/day respectively, blastocysts collected 5 days after mating; Text-figure 12). Inclusion of ethanol (1.2%; v/v) in the culture medium in Experiment 3 (added to provide an appropriate control to the Trilostane supplemented medium) may thus suppress release of C21 steroids by blastocysts in vitro. This possible effect, also, was not investigated further.

The results of this study provide direct evidence for secretion of oestrogens by intact pig blastocysts between Days 3 and 7 after fertilization. Previously, definitive proof for aromatization in pig blastocysts before Day 10 after fertilization was not available (Flint et al., 1979). Oestrogens have, however, been implicated in initiation of blastocyst spacing (Pope, Maurer & Stormshak, 1982), stimulation of secretion of protein, calcium and prostaglandins from the endometrium
(Geisert et al., 1982a), luteotrophic effects (Gardner, First & Casida, 1963; Bazer & Thatcher, 1977; Frank et al., 1978; Ford & Magness, 1980), effects on uterine blood flow (Dickson, Bosc & Locatelli, 1969; Bazer, Roberts & Sharp, 1978; Ford, Christenson & Ford, 1982), and other effects consistent with maternal recognition and maintenance of pregnancy (Heap et al., 1977). The present results show that oestrogens of blastocyst origin may influence these functions prior to Day 7 after fertilization. Reports of impaired implantation (Bhatt & Bullock, 1974) and intra-uterine mortality and degenerative changes (Dey, Dickmann & Sen-Gupta, 1976) in rabbit blastocysts treated with an anti-oestrogen provides further circumstantial evidence for the significance of de novo oestrogen synthesis in mammalian blastocysts.

In the present study, oestradiol was secreted by Day 3 and Day 6 blastocysts at a higher rate relative to oestrone (Table 20), supporting previous indirect evidence for oestradiol being the main unconjugated oestrogen of conceptus origin contributing to histotroph (Chapter 3.2.4) and to the uterine venous plasma (Ford, Christenson & Ford, 1982) in early pregnancy. Rates of secretion of both oestrogens were depressed relative to androstenedione and testosterone (Table 20).

Within the control groups (Table 20), higher rates of steroid secretion by embryos cultured from Day 6 after fertilization, relative to Day 3, are assumed to reflect a higher metabolic (enzyme) capacity associated with increased protein content of the expanded Day 6 blastocysts (Wright et al., 1983).

The present study quantitates release of 6 steroids by preimplantation embryos of the pig. Secretion and concentration of steroids by blastocysts is consistent with modification of the intra-uterine milieu to provide those growth factors which are essential to preimplantation development, and with preparation of the endometrium for accommodation of the trophectoderm at implantation. The significance of a capacity for de novo synthesis and release of steroids by preimplantation blastocysts is now self-evident, and
our results demonstrate this capacity in preimplantation pig blastocysts cultured in the presence of authentic pregnenolone sulphate. In view of the abundance of pregnenolone sulphate in uterine fluid of early pregnant sows (Table 4), the results support earlier speculation that this substance serves a potentially significant role as a precursor to steroid synthesis by pre-implantation pig embryos in vivo (ibid). Recent evidence for high levels of pregnenolone sulphate in uterine washings of women between Days 15 and 28 of the menstrual cycle (Chapter 5.5; Stone, Petrucco & Seamark, 1984) suggests that pig blastocysts may not be unique in this regard.

The present results provide no evidence to suggest that in vitro development of pig blastocysts is affected by supplementation of culture media with precursors to, or inhibitors of, steroidogenesis.
Initially, the effects of supplementation of synthetic culture media with different energy and protein sources on *in vitro* development of pig embryos beyond the 4-8 cell stage were explored.

Minimal Essential medium (MEM) supplemented with glucose (1 mg/ml) proved superior to Kreb's-Ringer Bicarbonate (KRB) supplemented with glucose in its capacity to support *in vitro* development of pig embryos to the expanded blastocyst stage (*P*<0.05). Inclusion of pyruvate (0.25 mM) or lactate (25 mM) in either MEM or KRB based media inhibited embryonic development. As pyruvate and lactate are important and readily utilizable energy sources for development of most other mammalian embryos *in vitro*, it was suggested that the observed inhibitory effects of these substances reflect comparatively lower critical ranges of concentrations of pyruvate and lactate for optimum development of pig embryos *in vitro*.

As a supplementary protein source to MEM, heat-inactivated (HI) human serum (10% v/v) was superior (*P*<0.05) to HI-pig serum (10% v/v), HI-foetal calf serum (10% v/v) or bovine serum albumin (5 mg/ml). The proportion of 4-8 cell pig embryos which developed beyond hatching in MEM supplemented with HI-human serum (>56%) was higher than any other reported for *in vitro* culture of pig embryos through the same developmental period.

When pig blastocysts were cultured in this medium, supplemented with authentic pregnenolone sulphate, pregnenolone and progesterone were the predominant secretory products. The amount of each of these steroids secreted by blastocysts increased (from 0.40 and 0.07 pmol/day respectively, to 1.7 and 0.4 pmol/day respectively) when the concentration of pregnenolone sulphate in the culture medium was increased from 0.01 μM to 4.5 μM.

Secretion of progesterone by cultured blastocysts was suppressed when culture media were supplemented with Trilostane, an inhibitor of 3-β HSD. Together with the evidence for elevated concentrations of pregnenolone and progesterone in blastocele fluid (about 4 and 15 μM respectively; Table
19), and higher total contents of these steroids in expanded blastocysts, the results indicate synthesis and accumulation of C\textsubscript{21} steroids by pig blastocysts \textit{in utero}.

Secretion of androstenedione, testosterone, oestradiol and oestrone by preimplantation pig embryos was also demonstrated. The higher rate of secretion of oestradiol, relative to oestrone, was consistent with other evidence that oestradiol is the main unconjugated oestrogen secreted by conceptus tissue in the pig \textit{in vivo}. Finally, these data provide direct evidence for secretion of oestrogens by intact pig blastocysts between Days 3 and 7 after fertilization, and support earlier speculation that pregnenolone sulphate serves a potentially important role as a precursor to steroid synthesis by preimplantation pig embryos \textit{in vivo} (as proposed in Chapter 3.2).
5.5 LEVELS OF STEROID HORMONES IN WASHINGS OF THE HUMAN UTERUS DURING THE MENSTRUAL CYCLE

Introduction

About 40% of fertilized human ova reabsorb during the first two weeks of pregnancy (Hertig, Rock, Adams & Menkin, 1952). Of those embryos which successfully implant, 37% are lost before 12 weeks of gestation (Edmonds, Lindsay, Miller, Williamson & Wood, 1982), 43% are lost before 20 weeks of gestation (Miller, Williamson, Glue, Gordon, Grudzinkas & Sykes, 1980), and about 12% abort during the final 18 weeks of pregnancy (Edmonds et al., 1982). The total embryonic loss during human pregnancy is thus near 73%, which agrees closely with the statistical estimate of 78% by Roberts and Lowe (1975), and is consistent with about 25% of transferred, in vitro fertilized (IVF) embryos being maintained to term. About 30% of this embryonic loss will encompass elimination of genotypic variants (e.g. errors of gametogenesis, defects of fertilization, mosaicism, differentiation failure; Nishimura, 1970), but causes for the remaining 70% of post-fertilization wastage in the human remain poorly understood.

A component of the high level of early embryonic loss during human pregnancy may reflect constraints to embryo nutrition imposed through deficiencies in endometrial secretion before the time of implantation, and deficiencies in histotroph and in haemotroph after implantation. In this respect, human embryonic tissues may have a capacity to release steroids which are essential to the maintenance of an intra-uterine environment conducive to expansion of embryos, and their implantation. Measurement of steroids in uterine fluids of pigs identified potentially important precursors to steroidogenic activity by intact blastocysts of that species (Chapters 3, 4 and 5). In view of the likelihood that human uterine fluids
and embryos may exhibit similar properties, the study detailed below describes the occurrence of steroid hormones in human uterine washings.

Materials and methods

Saline (0.9%, w/v; 10 ml) washings were collected from uteri of 73 women undergoing tubal surgery. The fallopian tubes of these women were previously confirmed to be occluded, allowing quantitative recovery of flush volumes which exceed the normal volume of the human uterine lumen (about 500 μL).

Levels of 10 steroids in the washings were assayed using the chromatographic and radioimmunoassay procedures detailed in Chapter 3.2.2.

Results

Total levels of oestrone in all collections were <0.9 pmoles. Total levels of the other steroids in washings of individual uteri are plotted against the number of days lapsed since each patient's last menstrual period (LMP) in Text-figure 14.

The average total levels of DHEA sulphate in washings of uteri during the 14 days preceding menstruation (595 pmoles/wash) exceeded (P<0.1) the respective average value for Days 1 to 14 after menstruation. There was no other evidence for variation in the steroid content of uterine washings during the menstrual cycle (Text-figure 14; Table 21).

Average total levels of the 10 steroids in human uterine washings are compared in Text-figure 15, DHEA sulphate and pregnenolone sulphate being the predominant steroid components of this milieu (Text-figure 15).

Discussion

The predominance of DHEA sulphate and pregnenolone sulphate in washings of
Text-figure 14: Total levels of steroid hormones in washings of uteri of women during the menstrual cycle. All values for total oestrone were <0.9 pmoles.

Average (± s.e.m.) values for each steroid are shown toward the right-hand side of each abscissa (X).

Values to the far right of each abscissa represent steroid levels in washings of uteri of women experiencing amenorrhea (●) or oligomenorrhea (▲).
Table 21: Total content of steroids in human uterine washings (mean ± s.e.m.; pmol)

<table>
<thead>
<tr>
<th>Steroid</th>
<th>Days after last menstrual period</th>
<th>Difference (P value)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 - 14 (N=42)</td>
<td>15 - 28 (N=31)</td>
</tr>
<tr>
<td>C₂₁</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pregnenolone</td>
<td>6.57 ± 1.06</td>
<td>5.31 ± 0.99</td>
</tr>
<tr>
<td>Pregnenolone sulphate</td>
<td>29.11 ± 6.16</td>
<td>18.53 ± 6.87</td>
</tr>
<tr>
<td>Progesterone</td>
<td>3.17 ± 0.94</td>
<td>2.51 ± 0.43</td>
</tr>
<tr>
<td>C₁₉</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DHEA</td>
<td>18.26 ± 3.25</td>
<td>14.98 ± 2.71</td>
</tr>
<tr>
<td>DHEA sulphate</td>
<td>595.35 ± 77.63</td>
<td>401.84 ± 73.04</td>
</tr>
<tr>
<td>Androstenedione</td>
<td>2.62 ± 0.33</td>
<td>3.62 ± 1.09</td>
</tr>
<tr>
<td>Testosterone</td>
<td>0.96 ± 0.14</td>
<td>0.71 ± 0.12</td>
</tr>
<tr>
<td>C₁₈</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oestrone</td>
<td>0.9</td>
<td>0.9</td>
</tr>
<tr>
<td>Oestrone sulphate</td>
<td>0.84 ± 0.15</td>
<td>0.56 ± 0.15</td>
</tr>
<tr>
<td>Oestradiol</td>
<td>0.16 ± 0.05</td>
<td>0.08 ± 0.02</td>
</tr>
</tbody>
</table>
Text-figure 15: Average (± s.e.m.) total levels of sex steroids, and their precursors, in washings of human uteri throughout the menstrual cycle (N = 68; amenorrheic and oligomenorrhoeic values excluded).
human uteri during the menstrual cycle is in accord with the previous analyses of uterine fluids from non-pregnant pigs (Table 4). If human blastocysts exhibit aryl sulphatase activity and have the capacity to synthesize oestrogens from pregnenolone or DHEA, as do blastocyst tissues from other mammals studied (e.g. Flint et al., 1979; Heap et al., 1981; Chapter 5.3), these steroid sulphonyl conjugates are potentially important precursors for synthesis of progestagens, androgens and oestrogens by human blastocysts in vivo.

In regard to the integrity of human embryos grown in vitro and in vivo, these mechanisms provide for synthesis of steroids which would, directly or indirectly, stimulate embryonic expansion and development. For example, progesterone could provide immune cytoprotection for embryos (Siiteri et al., 1977; Fujisaki et al., 1985), induce endometrial enzyme activity (e.g. Clarke, Adams & Wren, 1982) or stimulate endometrial protein secretion (e.g. Hirsch, Fergusson & King, 1977). Androgens could act locally as protein anabolic agents.

While the steroid sulphates (or their conjugates) accumulated in uterine fluids of sows between Days 9 and 15 after oestrus appear to originate from ovarian tissues (Chapters 3 and 4), DHEA sulphate and pregnenolone sulphate accumulated in human uterine fluids are more likely to originate as adrenal secretions (Arcos et al., 1964).

The present study provides no evidence for substantial changes in the steroid composition of human uterine secretions during the menstrual cycle.
CHAPTER VI

RELEASE OF PROSTAGLANDINS BY CULTURED PIG BLASTOCYSTS

6.1 INTRODUCTION

Prostaglandins have been associated with embryo migration (Pope, Maurer & Stormshak, 1982), spacing (Kennedy, 1977), expansion (Biggers, Leonov, Barker & Fried, 1978), elongation (Davis, Pakrasi & Dey, 1983), vascular changes at implantation (Kennedy & Armstrong, 1981), and implantation (Kennedy, 1977; Evans & Kennedy, 1978; Kennedy & Zamecnik, 1978; Biggers et al., 1978; Johnson & Dey, 1980; Saksena, Lau & Chang, 1976; Hoffman, 1978) and have been implicated in mechanisms of early recognition of pregnancy (Marcus, 1981; Davis et al., 1983). Furthermore, administration of inhibitors of PG synthesis (e.g. indomethacin) during early pregnancy inhibits blastocyst expansion and hatching (Hurst & MacFarlane, 1981) and implantation (El-Banna, Sacher & Schilling, 1976; Hurst & MacFarlane, 1981), and is associated with degeneration and resorption of foetuses (El-Banna et al., 1976; Hoffman, 1978). The indomethacin-induced block of implantation is reversible by subcutaneous injection of PGE2 or PGF2-α (Lau, Saksena & Chang, 1975; Saksena et al., 1976; Kennedy, 1977). Implantation of mammalian embryos is preceded by an increase in permeability of endometrial capillaries at sites where blastocysts are present (Psychoyos, 1973). As PGs have been implicated in increased tissue permeability in inflammatory responses of other tissues (Williams & Morley, 1973), the increase in endometrial vascular permeability preceding implantation may also be mediated by PGs. In this respect, indomethacin blocks the increase in permeability of endometrial capillaries, and suppresses the blueing response.
due to leakage of injected Evan's blue dye at implantation sites in the rat (Kennedy, 1977; Hoffman, 1978; Hoffman, DiPietro & McKenna, 1978). Concentrations of prostaglandins E and F are usually higher in the Evan's blue dye sites compared with other areas of the endometrium (Kennedy, 1977).

Mechanisms of action of PGs in early embryonic development may involve the blastocyst (Biggers et al., 1978; Holmes & Gordashko, 1980), uterine tissues (Evans & Kennedy, 1978; Jonsson, Rankin, Ledford & Baggett, 1979; Rankin, Ledford, Jonsson & Baggett, 1979; Lundkvist & Nilsson, 1980), or both. Synthesis and release of PGs by blastocysts in vitro has been demonstrated for cows (Shemesh, Milagiv, Ayalon & Hansel, 1979; Shemesh & Hansel, 1983), rabbits (Dey, Chien, Cox & Crist, 1980; Harper, Norris & Rajkumar, 1983; Racowsky & Biggers, 1983; Jones & Harper, 1984), mice (Chepenik & Smith, 1980) and sheep (Hyland, Manns & Humphrey, 1982), but not for the rat (Kennedy & Armstrong, 1981). In the pig, dispersed tissues from elongated blastocysts (Day 16 after fertilization) can exhibit a capacity to metabolize arachidonic acid (Lewis & Waterman, 1982) and to synthesise PGF (Watson & Patek, 1979), consistent with an increase in the PGF and PGF-A content of pig blastocysts between Days 7 and 14 after coitus, and a corresponding increase in phospholipase activity (Davis et al., 1983).

The capacity for synthesis of PGs by intact preimplantation embryos in the pig has not previously been investigated. The present study examines the release of PGs E2, F2-α and H2 by pig blastocysts in vitro, preliminary to future studies on roles of prostanoids in preimplantation development and implantation in this species.
6.2 MATERIALS AND METHODS

Collection of embryos

Embryos were obtained from Large-White sows mated four days previously. Sows were anaesthetized (Fluothane; N₂O; O₂ as detailed in Chapter 4.1.2) and the uteri exposed by mid-ventral laparotomy. The embryo flushing procedure used has been detailed previously (Stone et al., 1984a). Sterile precautions were observed throughout surgery and during embryo handling.

Embryo handling

Ten embryos recovered from the uterus were washed twice in the transport medium and twice in culture medium. The culture medium was based on MEM, and was supplemented with 25 mM sodium bicarbonate and 10% (v/v) heat inactivated human serum (Chapter 5.2.2).

Washed, morphologically normal, embryos were transferred to 500 ul of culture medium in a glass tube which was subsequently capped, vented and equilibrated at 37°C in an atmosphere of 5% CO₂; 5% O₂; 90% N₂.

A control tube, without embryos, was set up in the same manner.

Sampling

For 8 consecutive days, commencing on the day of embryo recovery, 3x100 ul aliquots of culture medium were carefully aspirated from the top of each blastocyst culture and control tube every ten minutes between 1400 and 1500 h. Volumes of medium removed were replaced with 300 ul of fresh, equilibrated culture medium.

The first 100 ul aliquots were maintained at room temperature for 1h, then frozen (-15°C) until assayed for PGF₂-α.
The second aliquot was used in the assessment of PGH2 release. PGH2, an endoperoxide, is an unstable intermediate in PG synthesis and, at room temperature, decomposes to PGE2 and PGF2-α in the approximate ratio of 4:1 (R.W. Kelly, personal communication). Any PGH2 released by blastocysts will thus appear in these aliquots of culture medium as the metabolites, PGF2-α and PGE2, and contribute to the total levels of these substances measured in the culture medium. In the presence of an inhibitor of isomerase activity, PGH2 preferentially degrades to PGF2-α and, by measuring levels of PGF2-α in isomerase-active and isomerase-inhibited media, levels of PGH2 in the original medium can be estimated. For this reason, the second 100 ul aliquot was dispensed directly into 11 ul 55% (w/v) stannous chloride in ethanol to inhibit isomerase (Hamberg & Samuelson, 1974), and stored (-15°C) for PGF2-α radioimmunoassay. The molar concentration of PGH2 in freshly collected culture medium was calculated according to \((d-c)x1.25\), where 'c' and 'd' are the molar concentrations of PGF2-α in the first and second aliquots respectively. The molar concentrations of PGE2 and of PGF2-α present in the original medium as a result of PGH2 decay then equal \((d-c)\) and \([((d-c)/4]\) respectively.

The third 100 ul aliquot of culture medium was retained for assay of PGE2 as the methoxime, and was dispensed directly into a mixture of 250 ul methoxyamine hydrochloride in water (160 mg/ml) and 1 ml sodium acetate buffer (1.5 M, pH 5.1). The mixture was briefly vortexed and tubes were incubated at 60°C for 1h, to convert any PGE2 present in the original culture medium to a stable PGE2 conjugate, PGE2-methyloxime (Kelly, Deam, Cameron & Seamark, 1985).

All solutions were stored at -15°C and levels of the methoxymated PGE determined by radioimmunoassay (ibid).
PG radioimmunoassay

PGF2-α. Duplicate aliquots of culture medium (40 ul) were extracted with 2 ml ether : ethyl acetate (3:1). Levels of PGF2-α in the dried extracts were determined according to the method detailed by Liggins, Campos, Roberts & Skinner (1980), substituting a higher specific activity tracer [PGF2-α (5,6,8,9,11,12,14,15-3H); New England Nuclear, specific activity 150 Ci/mmol] and using a final antiserum dilution of 1:40000. The antiserum was raised in rabbits against PGF2-α conjugated with bovine serum albumin, and was obtained from Professor G.C.Liggins, University of Auckland, New Zealand. This antiserum cross-reacted 22.6% with PGF1-α, 1.8% with 13,14-dihydro-PGF2-α, 1.6% with 13,14-dihydro-PGF1-α, and <1.0% with 17 other PG-related compounds tested (Liggins et al., 1980). All samples were assayed in a single assay, the mean intra-assay coefficient of variation being 8%. The sensitivity of the assay was 0.07 pmoles PGF2-α.

PGE2-methyloxime. Duplicate 500 ul aliquots of methyloximated culture medium (equivalent to 37 ul of original culture medium) were extracted with 2.5 ml ether : ethyl acetate (3:1). Extracts were washed with 2 ml distilled water and dried under air at 37°C. PGE2-methyloxime tracer was synthesized from PGE2 [5,6,8,11,12,14,15-3H(N)] (New England Nuclear; Cat.# NET 428) according to the protocols described for methoximation of PGE2 in culture medium (the methodology and reagents for synthesis, extraction and radioimmunoassay of PGE2-methyloxime in the present study were kindly made available by Dr. R.W. Kelly and Sylvia Deam). The PGE2-methyloxime antiserum was raised in goats against PGE2-methyloxime conjugated to bovine gamma-globulin. Used at a final dilution of 1:40000, the cross-reactivities of the antiserum were 13% with PGE2, 0.47% with 15-keto-PGE2-methyloxime, 0.20% with PGA2-methyloxime, 0.18% with 13,14-dihydro-15-keto-PGE2-methyloxime, and <0.08% with PGA2, PGF2-α, 13,14-dihydro-PGF2-α-methyloxime, 13,14-dihydro-15,keto-PGE2, 15,keto-PGE2.
and 13,14-dihydro-PGF2-\( \alpha \). All samples were assayed in a single assay, the mean intra-assay coefficient of variation being <8%. The sensitivity of the assay was 0.02 pmol PGE2-methyloxime.

6.3 RESULTS

Levels of PGE2 and of PGF2-\( \alpha \) in respective aliquots were determined, and levels of PGH2 calculated according to the formula defined above. Levels of PGF2-\( \alpha \) and PGE2 measured in aliquots 1 and 3 respectively were corrected for effects of PGH2 decay.

Rates of release of PGE2, PGF2-\( \alpha \) and H2 during the one-hour sampling periods of each of the 8 days of culture were calculated from the total level of each PG in the blastocyst culture tube, corrected for the amount calculated to be present ten minutes previously, and corrected for levels in the control tube. Mean (\( \pm \) s.e.m.; \( N=6 \)) rates of release of each PG between Days 4 and 11 after fertilization are shown in Text-figure 16.

6.4 DISCUSSION

Of the three PGs determined in this study, only PGE2 was released by blastocysts at a significant rate (about 22 pmol/blastocyst/day), rates of release of PGH2 and of PGF2-\( \alpha \) being below 4 pmol/blastocyst/day.

Previous reports by Lewis & Waterman (1978), Watson & Patek (1979) and Davis et al. (1983) have demonstrated the capacity for synthesis and storage of PGF2-alpha by pig blastocysts. In view of the reported vasoconstrictive properties of PGF2-\( \alpha \) (DuCharme & Weeks, 1967; Greenberg & Sparks, 1969; Hodgman, Jelks, Swindall & Daugherty, 1970; Powell & Brody, 1973) and current understanding of changes in the uterine endometrium around the time of implantation (Chapter 2.2), release of PGF2-\( \alpha \) by blastocysts would appear to oppose normal changes in the endometrium at implantation. The results of this study indicate that, if PGF2-\( \alpha \) is synthesized by pig
Text-figure 16: Release of prostaglandins E, F2-α, and H by cultured Day-4 pig blastocysts. Values shown are average (± s.e.m.) rates determined from 7 consecutive 10-minute samplings of culture medium, on each of the 8 consecutive days of culture (as detailed in the methods).

To convert ordinate values to pmol/blastocyst/day, multiply by 14.4. Respective limits of sensitivity of the prostaglandin assays (pmol/tube) are indicated (Δ).
blastocysts, it is not released at substantial rates. The significance of de novo synthesis and storage of PGF2-α by blastocysts remains speculative (Jones & Harper, 1984).

In regard to the origins of prostaglandins released by blastocysts, evidence now suggests that prostaglandins accumulated by mammalian blastocysts originate from the intra-uterine milieu. For example, Jones & Harper (1984) demonstrated passive uptake and efflux of prostaglandins (E and F) by preimplantation rabbit blastocysts, with no apparent saturation of uptake over a PG concentration range up to 1 μM. Levels of PGE2 rise, and levels of PGF2-α fall, in histotroph of pregnant rabbits after Day 4 post coitum (Sharma, 1979), so passive exchange of PGs between histotroph and blastocoelic fluid, and greater uptake of PGs by more advanced blastocysts in vivo (Jones & Harper, 1984), should then result in a rising PGE/PGF ratio in blastocoele fluid of rabbit embryos. This is consistent with proportionately greater release of PGE by rabbit blastocysts on Day 7 after fertilization (ibid). There was no evidence for PG catabolism in Jones & Harper's study. Furthermore, sheep blastocysts accumulate PGE between Days 13 and 15 after coitus, during which time PGE is preferentially released to histotroph (Hyland & Manns, 1982).

In view of this evidence for passive exchange of PGs between blastocoelic fluid and the surrounding milieu by rabbit and sheep embryos, and recent indications that levels of PGE in histotroph of sows between Days 10 and 12 of gestation are about 5x higher than PGF levels (Geisert, Thatcher, Roberts & Bazer, 1982), the higher rate of PGE release (relative to PGF; Figure 16) in the present study may reflect release of PGs which were passively incorporated into the pig blastocysts prior to their collection. Future experiments examining PG release by pig blastocysts cultured in media supplemented with prostaglandin synthetase inhibitors (e.g. Indomethacin) will indicate the contribution of prostaglandin synthesis to this prostaglandin release in vitro.

There is compelling data to now support the view that PGs play a central
role in early pregnancy and in implantation (e.g. Chapter 6.1). On the basis of identification of PGs in blastocysts of a number of species, it has been proposed that the blastocyst releases PGs and exposes endometrial tissues to high local PG concentrations. The results of this study, being the first to quantitate the capacity for PG secretion by intact preimplantation pig blastocysts, supports this view with particular respect to PGE2. Furthermore, evidence that the rate of release of PGE2 exceeds that of PGF2-α, and of PGH2, is significant in view of the specific capacity for PGE2 to increase endometrial vascular permeability indices (Kennedy, Barbe & Evans, 1980); PGF2-α is vasoconstrictive (DuCharme & Weeks, 1967; Greenberg & Sparks, 1969; Hodgman et al., 1970; Powell & Brody, 1973) and would thus tend to impair effective implantation.

PGE2 also has antiluteolytic activity (Inskeep & Murdoch, 1980), so the release of PGE by preimplantation blastocysts of the pig may be implicated in the maternal recognition of pregnancy, occurring between Days 10 and 12 after mating (Dhindsa & Dziuk, 1968). The onset of oestrogen synthesis by pig blastocyst tissue occurs near Day 10 after mating (Flint et al., 1979; Chapter 3.2) and would, on the basis of the results of the present study and of Davis et al. (1983), be preceded by the release of PGE by 3 to 6 days. In view of the capacity for PGs of the E series to bind specifically to cell receptors and stimulate adenyl cyclase activity (LeMaire & Marsh, 1975), PGs of blastocyst origin may thus be involved in the initiation of steroid hormone production; an opposing view to that proposed previously by Pakrasi & Dey (1983).

The capacity for preimplantation pig blastocysts to secrete PGs and steroid hormones (particularly oestrogens) have now been demonstrated and roles for PGE and oestrogens in the process of implantation have been defined. Interactions between regulatory effects of specific steroids and PGs during preimplantation development remain to be elucidated. Similarly, further studies are required to determine whether PGs released by pig blastocysts are synthesized by blastocyst tissues, or are accumulated from
the intra-uterine milieu, as can occur in rabbit blastocysts (Jones & Harper, 1984).

Blastocysts cultured in the present study hatched from their zonae between Days 6 and 8 after fertilization. Three of these blastocysts degraded thereafter, as indicated by reducing trophoblast diameters. It is thus assumed that the rise in PGF2-α release, and fall in PGE release, which coincided with these changes (Text-figure 16) reflects necrosis of the cultured conceptus tissues, rather than normal prostaglandin release by pig blastocysts after Day 9 post fertilization.

6.5 SUMMARY

The rate of release of PGE2 by cultured pig blastocysts (about 0.15 pmol/blastocyst/10 min) exceeded respective rates of release of PGF2-α and PGH2 by about 5x. In regard to roles for blastocyst-derived PGs in implantation in the pig, this evidence is significant as PGE2 can increase endometrial vascular permeability. Blastocyst-derived PGE may also be implicated in the recognition of pregnancy by the sow.
CHAPTER VII

PRACTICAL ASPECTS

7.1 IDENTIFICATION OF LOW FECUNDITY SOWS BY ANALYSING LEVELS OF OESTRONE SULPHATE IN PLASMA

Introduction

During early pregnancy in the pig, oestrone originating in conceptus tissues (Perry, Heap & Amoroso, 1973; Perry et al., 1976; see Chapter 5) is sulfoconjugated in uterine tissues (Pack & Brooks, 1974; Pack et al., 1979; Dwyer & Robertson, 1980; Meyers et al., 1983) and is released to the uterine venous drainage as oestrone sulphate (Robertson & King, 1974). Peripheral plasma levels of oestrone sulphate in early pregnant sows exceed non-pregnancy values before Day 17 after oestrus (Robertson, King & Dyck, 1978; see Text-figure 6) and reach maximum values between Days 25 and 30 after mating (Robertson & King, 1974), coinciding with a peak in urinary oestrone levels (Lunaas, 1962; Raeside, 1963).

The conceptus is assumed to be the principal source of oestrone in early pregnant sows (Robertson & King, 1974) and, in this regard, there are published reports of correlations between the rate of urinary excretion of oestrogen on Day 27 after mating and litter size (Edgerton, Erb & Harrington, 1971), and between plasma oestrone sulphate levels from Days 20 through 26 of gestation and litter size at Day 32 after mating (Horne, Chew, Wiseman & Dziuk, 1983). If these studies could be extended to allow early prediction of litter size at term, non-pregnant sows, and sows carrying small litters, could be identified and culled within 30 days of mating.
In this regard, Cunningham, Hattersley & Wrathall (1983) reported a positive correlation between litter size at term and levels of oestrone sulphate in sow plasma, although the regression constructed from their results was not of predictive value. These authors did not, however, discriminate between days of blood sampling in the range 25 to 30 days after mating. Plasma oestrone levels change markedly during this period, reflecting increased oestrogen secretion by trophectoderm tissues before Day 29 of pregnancy (Robertson & King, 1974) and declining endometrial sulphotransferase activity thereafter (Dwyer & Robertson, 1980). Resultant fluctuations in plasma oestrone sulphate levels between Days 18 and 40 of pregnancy (near 30% variation between consecutive days; Robertson & King, 1974) would contribute to the wide variation in plasma oestrone sulphate values in the study of Cunningham et al. (1983), consistent with the absence of predictive value in their regression.

The present study examines relationships between litter size and oestrone sulphate levels in plasma collected from sows on discrete days between Day 22 and Day 33 after mating, the phase of early pregnancy during which levels of oestrone sulphate in plasma are highest.

Materials and methods

Collection of plasma samples. Blood samples were collected by ear vein puncture from each of 837 Large White x Landrace sows between Days 22 and 33 after mating inclusive. Samples were drawn directly into heparinized tubes and stored at 4°C before centrifugation (2011 g, 4°C, 20 min) and aspiration of plasma, about 24 h later. None of the sows sampled had shown behavioural oestrus between Days 18 and 22 after mating.

To establish a comparative range of oestrone sulphate levels in plasma of non-pregnant animals, further blood samples were collected from 23 unmated sows.
Oestrone sulphate radioimmunoassay. The general assay methods are detailed in Chapter 3.2.2. Aliquots of sample plasma (20 μl, in duplicate) were dispensed into polypropylene assay tubes. Evaporated standards were redissolved in 20 μl of steroid-free sow plasma in identical tubes. Tracer ([6,7-3H(N)]-oestrone sulphate, ammonium salt; 52.5 Ci/mmol. New England Nuclear, Boston, Massachusetts, U.S.A.) was added to standard and sample tubes (9000 dpm in 100 μl assay buffer per tube) and equilibrated at 37°C for 10 min.

The antiserum used was raised in goats against oestrone-3-carboxyethylacetate coupled to BSA. The cross-reactivity of this antiserum, used at a final dilution of 1:24000 and incubated with antigen under conditions used in the assay (37°C for 15 min, 4°C for 1 h), was 320% with oestrone, 0.52% with oestradiol, 0.27% with progesterone, 0.05% with androstenedione, 0.032% with testosterone, 0.031% with oestriol, 0.020% with DHEA, 0.017% with DHEA sulphate, 0.002% with pregnenolone sulphate and <0.001% with pregnenolone. Despite the high cross-reactivity of this antiserum with oestrone, levels of this steroid in plasma of sows during the phase of pregnancy investigated are about 0.44% of the plasma oestrone sulphate concentrations (Robertson & King, 1974) and oestrone cross-reactivity would thus account for <1.5% of the estimated oestrone sulphate level in plasma.

Samples were assayed in duplicate, the average intra-assay coefficients of variation being less than 10%. On the basis of determinations of oestrone sulphate in aliquots of pools of sow plasma containing 4.8, 15.2, 33.5 and 36.2 nM oestrone sulphate, the inter-assay coefficient of variation of the assay is <6.9%.

Statistics. Relationships between total litter size and levels of oestrone sulphate in plasma (nM) collected from sows on discrete days after mating (Days 22 through 33) were analysed by linear regression (Snedecor & Cochran, 1980).
Results

Using the direct radioimmunoassay described, average (± s.e.m.) oestrone sulphate concentrations in plasma of un-mated sows between Days 1 and 11 (N=10), and between Days 11 and 21 (N=13), of the cycle were 0.80 (± 0.2; range <0.50 to 1.88) nM and 0.83 (± 0.19; range <0.50 to 1.87) nM respectively.

For mated animals, linear regressions between the oestrone sulphate level in plasma and total litter size at term (Table 22) show significant (P<0.01) correlations between litter size and levels of oestrone sulphate in plasma collected from sows between Days 22 and 29 after mating. Average litter sizes of sows in which plasma oestrone sulphate levels were below set values of between 0 and 30 nM, on alternate days during this period, are shown in Text-figure 17. For those sows which were bled between Days 22 and 29 after mating, the plasma oestrone sulphate levels below which farrowing did not occur were higher than the upper limit of the range of values for unmated animals (about 1.9 nM), and are shown in Text-figure 18. These values represent the plasma oestrone sulphate limits below which sows could be diagnosed as 'non-farrowing', and rise as the day of blood-sampling advances from Day 22 (2 nM) to Day 29 (7 nM) after mating.

None of the 765 sows blood-tested between Days 22 and 29 after mating were observed to return to oestrus before Day 22 post coitum, and 148 of these sows did not farrow. Levels of oestrone sulphate in plasma of seventy (47%) of these 148 'delayed return to oestrus' sows were within the range for unmated animals; that is, below 1.9 nM. Levels of oestrone sulphate in plasma of a further 64 (43%) of the 148 non-return/non-farrowing sows fell between 1.9 nM and the limits defined for non-farrowing (Text-figure 18).

Average litter sizes of sows with plasma oestrone sulphate levels above discrete values on Days 23, 25, 27 and 29 after mating are shown in Text-figure 19.
Table 22: Definition of linear regressions between litter size (y) and oestrone sulphate levels in plasma (nmol x), where $y = a + bx$.

<table>
<thead>
<tr>
<th>Days after matings</th>
<th>Number of matings</th>
<th>Intercept (a)</th>
<th>Slope (b)</th>
<th>Correlation coeff. (r)</th>
<th>Significance of &quot;r&quot; (P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>22</td>
<td>80</td>
<td>6.27</td>
<td>0.104</td>
<td>0.338</td>
<td>0.0011</td>
</tr>
<tr>
<td>23</td>
<td>52</td>
<td>4.63</td>
<td>0.198</td>
<td>0.530</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>24</td>
<td>76</td>
<td>5.53</td>
<td>0.148</td>
<td>0.418</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>25</td>
<td>111</td>
<td>5.73</td>
<td>0.128</td>
<td>0.424</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>26</td>
<td>89</td>
<td>3.34</td>
<td>0.172</td>
<td>0.676</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>27</td>
<td>167</td>
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<td>0.172</td>
<td>0.673</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>28</td>
<td>140</td>
<td>5.33</td>
<td>0.124</td>
<td>0.506</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>29</td>
<td>50</td>
<td>3.09</td>
<td>0.193</td>
<td>0.696</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>30</td>
<td>17</td>
<td>7.91</td>
<td>0.026</td>
<td>0.165</td>
<td>0.2630</td>
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<td>0.224</td>
<td>0.520</td>
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</tr>
<tr>
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<td>19</td>
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<td>0.001</td>
<td>0.002</td>
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</tr>
<tr>
<td>33</td>
<td>18</td>
<td>5.80</td>
<td>0.516</td>
<td>0.341</td>
<td>0.0837</td>
</tr>
</tbody>
</table>
Text-figure 17: Average litter sizes farrowed by groups of sows with plasma oestrone sulphate levels below discrete values on Days 23 (●), 25 (▲), 27 (○) and 29 (△) after mating.
Text-figure 18: Plasma oestrone sulphate concentrations, between Days 22 and 29 after mating, below which all sows failed to farrow.
Text-figure 19: Average litter sizes farrowed by groups of sows with plasma oestrone sulphate levels above discrete values on Days 23 (●), 25 (▲), 27 (○) and 29 (△) after mating.
Discussion

Significant (P<0.01) correlations between litter size at term and levels of oestrone sulphate in plasma of sows on Days 22 through 29 after mating (Table 22) provide a basis for prediction of litter size early in gestation. This period coincides with that phase of early gestation when plasma oestrone sulphate levels are highest (Robertson & King, 1974). Levels of oestrone sulphate in sow plasma fall abruptly to baseline values after about Day 29 of gestation (Hattersley, Drane, Matthews & Wrathall, 1980; Robertson & King, 1974), consistent with the absence of statistically significant relationships between oestrone sulphate concentrations after Day 29 of pregnancy and numbers of foetuses at Day 32 (Horne et al., 1983), or with numbers of piglets farrowed at term (Table 22).

The effectiveness of non-pregnancy diagnoses derived from low oestrone sulphate levels in plasma of sows between Days 20 and 30 after mating has been reported previously (e.g. Robertson & King, 1974; Saba & Hattersley, 1981; Cunningham et al., 1983) and, on the basis of farrowing outcomes of mated sows in this study (Text-figures 17), plasma oestrone sulphate concentrations below which early-pregnant sows (Days 22 to 29 after mating) could be diagnosed as 'non-farrowing' were derived.

The effectiveness of identification of delayed return/non-farrowing animals on the basis of measurement of oestrone sulphate in plasma appears superior to diagnoses based on vaginal biopsy/histology and plasma progesterone measurement, in which most delayed return/non-pregnant sows are predicted to be pregnant (e.g. Williamson & Hennessy, 1975; Hennessy & Williamson, 1976). The advantages of plasma oestrone sulphate measurement in identification of non-pregnant animals is consistent with quantitative relationships between plasma levels of oestrone sulphate and the amount of conceptus tissue present at the time of blood sampling (Horne et al., 1983). Vaginal histology and plasma progesterone levels reflect the maternal
As the bulk of oestrone sulphate in plasma of early pregnant sows originates from conceptus tissues as oestrone (Robertson & King, 1974), plasma levels of oestrone sulphate above the nadir of the unmated range (1.9 nM) are consistent with the presence of steroidogenically active trophoblast tissue. The presence of steroid-secreting conceptus tissue at the time of blood sampling is, therefore, indicated in the 64 non-return/non-farrowing sows in which plasma oestrone sulphate levels lay between 1.9 nM and the respective non-farrowing limits shown in Text-figure 18. The masses of viable conceptus tissue in these sows would, however, be predicted to be low (Table 22; Horne et al., 1983) and, as oestrone sulphate is luteotrophic in sows (Gardner et al., 1963; Bazer & Thatcher, 1977; Frank et al., 1978; Ford & Magness, 1980), the failure by these animals to farrow could indicate that a minimum mass of trophoblast tissue is necessary to maintain luteal function during early pregnancy (see du Mesnil du Buisson, 1961; Dhindsa & Dziuk, 1968; du Mesnil du Buisson & Denamur, 1968), and that too few embryos in the uterus between Days 22 and 29 after mating may predispose those few embryos to reabsorption thereafter. Alternatively, embryonic tissues present at the time of blood sampling may have been degenerating as a result of earlier pregnancy failure.

Levels of oestrone sulphate in plasma of only 9.5% of all non-return/non-farrowing sows in this study exceeded the 'non-farrowing' limits shown in Text-figure 18, representing 2.2% of all sows in which plasma oestrone sulphate levels exceeded the respective limits. By applying these non-farrowing limit values to mated sows blood sampled between Days 22 and 29 after mating, animals within the dry sow population of a pig herd would have a >97% probability of farrowing.

The non-return/non-farrowing rate in this study herd (19%) was higher than previously reported levels in commercial piggeries (about 10%; e.g. Hennessy & Williamson, 1976). While determinants of this inefficiency remain to be elucidated, the interpretation of the plasma oestrone sulphate
data indicates that embryonic reabsorption may account for between 43% (proportion of all non-return/non-farrowing sows with plasma oestrone sulphate levels between 1.9 nM and the non-farrowing limits shown in Text-figure 18) and 90% (proportion of all non-farrowing/non-return sows with plasma oestrone sulphate levels below the non-farrowing limits) of the incidence of non-return/non-farrowing in this particular herd.

In a practical respect, the significant relationships between litter size and plasma oestrone sulphate levels in plasma shown in Table 22 allow identification of low fecundity sows between Days 22 and 29 after mating in any pig herd. By culling these sows early in the assumed pregnancy, economic gains can be made through immediate feed and accommodation savings. Furthermore, as the average litter size farrowed in a herd will increase when sows are culled on the basis of low estimated litter size, indirect economies are realized through more efficient use of housing.

In those pig herds with low litter sizes, managers should be encouraged to mate more sows and use low predicted litter sizes as a criterion for reducing this group to a number which can be accommodated during farrowing.

In the present study, the increases in average herd litter size which would have resulted from culling sows in which plasma oestrone sulphate levels were below discrete values are shown in Text-figure 19. While the steepest gains are associated with exclusion of non-farrowing sows, the calculated average litter size of sows at farrowing increased with increasing oestrone sulphate values beyond the limits shown in Text-figure 18, in accord with the relationships described in Table 22.

Measurement of oestrone sulphate levels in plasma of sows between Days 22 and 29 after mating can thus provide early identification of non-pregnant animals, prediction of litter size in sows which are pregnant, and diagnosis of low fecundity in the short term.
7.2 AN IMMUNOLOGICAL APPROACH TO INCREASING REPRODUCTIVE EFFICIENCY

7.2.1 Introduction

For efficient establishment and maintenance of pregnancy in mammals, the foetus should differ antigenically from the mother (Billington, 1964; James, 1965; Edidin, 1972). The foetal component of the placenta is then a homograft and a homograft reaction in the maternal immune system against the foetal tissue occurs.

Immunogenetic disparity between the conceptus and mother can, then, contribute to the magnitude of the local inflammatory response which occurs in the endometrium at the site of implantation (Beer & Billingham, 1970). This inflammatory response is characteristic of a delayed hypersensitivity reaction and can stimulate local angiogenesis which facilitates transportation of nutrients and blood cellular elements to the implantation site (Beer, Scott & Billingham, 1975).

Where immunogenetic disparity between the mother and foetus exists, the enhanced inflammatory response at implantation sites can thus promote both the implantation of fertilized ova and growth of the foeto-placental unit. In examining this phenomenon with mice, Billington (1964) showed that placental size tended to be greater when the mother and foetus differed antigenically, and demonstrated that the increase in size was not due to heterosis. More recent evidence has supported this apparent immunological heresy, viz.;

(i) Degrees of histo-incompatibility improve efficiency of human reproduction and viviparity (Komlos, Zamir, Joshua & Halbrecht, 1977).

(ii) Some trophoblast antigens are allotypic and are shared with leucocytes (Faulk, Temple, Lovins & Smith, 1978), suggesting that antigens (such as histo-compatibility antigens; HLA) shared
between male and female partners could account for trophoblast/lymphocyte cross-reactive (TLX) antigens being shared between the blastocyst and mother. In these cases, the TLX-compatible embryo might not stimulate a maternal inflammatory response or blocking factors, and the blastocyst might be rejected like any other group of foreign antigens implanted into the uterus. Consistent with this scenario, human couples in which the woman has habitual spontaneous abortion share more common HLA A, B, C, D and DR loci than couples of average fertility (Gerencer, Drazancic & Kuvacic, 1979; Beer, Quebberman, Ayers & Haines, 1981). Furthermore, Taylor & Faulk (1981) reported correction of spontaneous abortion in 3/4 women by repeated transfusion, throughout pregnancy, with leucocyte enriched plasma from at least 16 different erythrocyte-compatible donors. These authors attributed the response to stimulation of a TLX-antigen response, consistent with prevention of rejection of the embryo.

(iii) Chronic aborters do not produce inhibitors of cell-mediated immunity usually found in maternal blood during pregnancy (McIntyre & Faulk, 1979).

(iv) Transplantation antigens are absent in human trophoblast membranes, but discrete trophoblast antigens are present which could form the basis for immune reactions between the mother and foetus.

(v) Teleologically, a species favouring histo-incompatibility in reproduction is advantaged through preservation of genetic polymorphism (Johnson, 1982).

Cogent evidence now confirms that allogeneic pregnancies lead to the production of specific lymphocytic receptor cells by the mother, but these
cells are unreactive because of specific (blocking antibodies) and non-specific (endocrine) restraining factors within the placenta (Maroni & de Sousa, 1973). Furthermore, presensitization and the mother's natural covert response to allogeneic conceptuses affords allogeneic conceptuses a significant selective advantage over syngeneic conceptuses (Edidin, 1972) and the level of maternal reactivity to alien tissue antigens during pregnancy has been shown to be a significant determinant of the size and weight of placentae and, in rats, of the entire foeto-placental unit (Beer & Billingham, 1974). Origins of the paternal antigens which stimulate maternal immunity in pregnancy are currently under investigation. Foetal tissue antigens have been suggested (Beer & Billingham, 1976), while other studies have indicated that the response is induced by spermatozoa, and not by foetal antigens (Prehn, 1960; Lengerova & Vojtiskova, 1963; Beer & Billingham, 1974).

Presensitization of females against tissue antigens of future conceptuses can thus stimulate development of conceptus tissues (Palm, 1974) and has been associated with significant increases in litter size in mice (James, 1965), rats (Beer & Billingham, 1974) and in hamsters (Beer, Scott & Billingham, 1975). In these studies, solid tissue and monodisperse cellular allografts, such as those of skin, spleen, lymphoid cells or washed spermatozoa, were effective in primary immunization (see Beer, Billingham & Hoerr, 1971). Suspensions of such allogeneic cells can elicit an immunologically specific hypertrophy of the draining para-aortic lymph nodes and a state of systemic transplantation immunity (ibid).

Litter size in gilts can also be higher following presensitization to antigens of boar semen before and after puberty (Murray, Grifo & Parker, 1982), although litter sizes from post-pubertal animals inseminated with single doses of killed boar sperm in this short study were not significantly different from control values.

The experiment described below aims to determine whether presensitization of post-pubertal sows to spermatozoal antigens is effective
in increasing litter size in this species. The effectiveness of this approach to systemic sensitization against spermatozoal antigens, as evidenced by delayed hypersensitivity, has been established previously (Marcus, Soffer, Ben-David, Peleg & Nebel, 1973).

7.2.2 Materials and methods

**Animals.** Boars within the NPRU breeding herd, South Australian Department of Agriculture, are assigned priority ratings for each sow scheduled for mating, the ratings being assigned to minimize the level of inbreeding in litters farrowed (Stone, Heap & Stafford, 1976). As a result of this routine management practice at the NPRU, the identity of the boar to which a particular sow will be mated at her next mating can be reasonably predicted. Boars in the NPRU breeding herd are routinely trained for semen collection.

**Material for presensitization.** Ejaculates were collected from boars at the NPRU, using a gloved-hand technique (du Mesnil du Buisson & Signoret, 1970). Ejaculates were strained through sterile gauze, and sperm concentrations in the filtrates were determined using a haemocytometer. Aliquots of semen, containing $5 \times 10^9$ sperm, were stored at $-15^\circ C$.

Optimum conception rates in artificially inseminated sows are achieved with five milliard sperm (Stone, 1982), so it is assumed that this sperm number provides sufficient antigen to stimulate an effective LA response in the sow uterus during early pregnancy. Therefore, five milliard sperm should provide sufficient sperm antigen for a LA response in non-conceptual oestrous cycles.

**Presensitization.** One half the number of sows to be mated at each quarterly mating period were inseminated at the first post-weaning oestrus with $5 \times 10^9$ killed sperm from the boar to which each would subsequently be mated. Each inseminate volume was made up to 50 ml with sterile 0.9% (w/v)
saline, and was warmed to 37°C prior to insemination. Sows were inseminated intracervically by Melrose insemination catheters, obeying normal sterile precautions, and were mated naturally at the second oestrus after weaning. Control sows were not treated.

Blood samples were collected from all sows (peripheral ear vein) between Days 26 and 28 after mating, and litter sizes were predicted on the basis of oestrone sulphate levels in plasma (according to the regressions detailed in Table 22). Total numbers of piglets farrowed at term were recorded.

This experiment commenced in July 1984 and, to date, only small numbers of farrowing outcomes can be compared (about 20 farrowings per group).

Statistics. Differences in plasma oestrone sulphate levels, predicted litter sizes, and actual litter sizes, between control and treatment groups were analysed using a t test (Clarke, 1969).

When sufficient data are available (about 200 farrowings per group), numbers of piglets (live and stillborn) delivered by sows in the presensitized and control groups will be compared by analysis of covariance; with sow parity and litter inbreeding levels as covariates, sensitization as the factor.

7.2.3 Results

The results of this study are summarized in Table 23. Levels of oestrone sulphate in plasma of control sows between Days 26 and 28 after mating were similar to 'presensitized' values (P>0.05), and predicted litter sizes calculated from these values were also similar (P>0.05; Table 23). The average total litter size farrowed by presensitized sows (11.9) was higher than the control value (9.3; P<0.025).
Table 23: Comparison of plasma oestrone sulphate levels, predicted litter sizes, and actual litter sizes, between control sows and sows presensitized by insemination with frozen-thawed semen (average values, ± s.e.m.)

<table>
<thead>
<tr>
<th></th>
<th>Plasma oestrone sulphate (nM)</th>
<th>Predicted(#)</th>
<th>Actual</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>23.14 ± 5.12</td>
<td>7.76 ± 0.51</td>
<td>9.29 ± 0.86</td>
</tr>
<tr>
<td>Presensitized</td>
<td>22.49 ± 1.93</td>
<td>7.10 ± 0.40</td>
<td>11.85 ± 1.02</td>
</tr>
<tr>
<td>Significance of difference (+)</td>
<td>NS</td>
<td>NS</td>
<td>**</td>
</tr>
</tbody>
</table>

#; Calculated using regressions detailed in Table 22.

+; NS - not significant

** - \( P < 0.025 \)
7.2.4 Discussion

The present study shows that litter sizes in sows can be increased by about 2.5 piglets per litter \((P<0.025)\) by inseminating sows with a single dose of diluted, frozen-thawed, semen (containing \(5 \times 10^{9}\) spermatozoa) at the oestrus preceding natural mating. Presently, the mechanism which evokes this response in sows is not understood, beyond the speculation that insemination of sows with dead semen will 'presensitize' their reproductive tracts to spermatozoal antigens; and that presensitization affords embryos an advantage at implantation. Tracts of sows in the present study were not examined for signs of reaction to the insemination treatment, although observations on uteri of other species following single insemination of suspensions of sperm or other allogeneic material indicate inflammation, oedema and leukocyte infiltration of endometrial tissues (Beer, Billingham & Scott, 1975), sensitization (Dorsman, Tumboh-Oeri & Roberts, 1978), and a persistent capacity for presensitized uteri to respond to local re-exposure or challenge with the same cellular antigens (Beer et al., 1975). Parallel studies have not yet been carried out with pigs, but the existing evidence suggests that tracts of sows treated in the present study would have responded immunologically to the insemination of frozen/thawed semen, and would be sensitive to the same seminal antigens at the time of natural mating (about 21 days after treatment). Increased litter sizes from treated sows in the present study is thus in accord with responses to uterine presensitization in laboratory animals, and with the preliminary indications for pigs by Murray et al. (1982).

Oestrone sulphate levels in maternal plasma can be equated with the steroidogenic activity of trophoblast tissue (see Chapter 7.1) and have been correlated with numbers of foetuses at about Day 32 of gestation (Horne et al., 1983) and at term (Stone, Seamark, Godfrey & Lloyd, 1985; Chapter 7.1). Plasma oestrone sulphate levels and predicted litter sizes in treated and in control sows in the present study were similar (Table 23), indicating
similar masses of functional trophoderm tissue in both groups of animals. In this respect, higher numbers of foetuses farrowed by treated sows suggests that fewer foetuses reabsorbed after Day 28 of pregnancy in the 'presensitized' sows, or that more embryos may have been present in the treated sows at the time of blood collection, each of these embryos being associated with a smaller average mass of trophoderm tissue. Regardless, the results are consistent with improved reproductive efficiency of sows sensitized to the antigens expressed in frozen-thawed semen.

These preliminary results show that reproductive efficiency and litter sizes in sows can be increased (by about 28%) by insemination of frozen-thawed semen at the oestrus preceding mating. The presensitization method used in this study is simple, does not require expensive or specialized facilities, and can be adopted legally by any competent herdsman.

Further studies are required to determine:

1. which component(s) of semen evoke the response described,
2. whether other alloantigens (e.g. blood cells) elicit a better response,
3. whether the frequency (and/or timing) of presensitization is related to the level of improvement in reproductive efficiency.
7.3 TRANSFER OF EMBRYOS COLLECTED FROM SLAUGHTERED SOWS

7.3.1 Introduction

The advantages of introducing new genetic material into specific-pathogen-free (SPF) pig herds as embryos, rather than as live animals, are readily appreciated. These include reduced risk of introducing disease (Martin, 1983) and cost-savings associated with transfer of zygotes. Current techniques for introducing genetic material through hysterectomy require transport and quarantine housing of pregnant sows prior to hysterectomy, are dependent upon successful cross-suckling of the hysterectomy litter onto a foster mother and, after removal of the gravid uterus, the monetary value of the carcase of the donor sow is not recovered. Embryo transfer offers advantages over hysterectomy in all these areas.

Furthermore, in relation to roles for embryo transfer in disease control and eradication programs, few viruses have been implicated in the infection of gametes (Eaglesome, Hare & Singh, 1980) and it is likely that most perinatal infections originate from the uterine environment. With early embryos (up to Day 6 post-fertilization), the zona pellucida which encases the morula and the blastocyst stages is impenetrable to most micro-organisms, including viruses (Eaglesome et al., 1980). The unhatched embryo is, therefore, protected against infection by micro-organisms, including porcine parvovirus, which is consistent with field evidence that parvovirus does not infect pig embryos (Wrathall & Mengeling, 1979 a, b).

The first successful embryo transfer in pigs in Australia was reported in 1980 at the NPRU. Since then, a further 15 successful transfers have been carried out at the NPRU, all involving surgical transfer between synchronized gilts.

If embryo transfer is to be used in the exchange of genetic material between pig herds, collection of embryos from donors following abattoir...
slaughter would be less wasteful, cheaper and simpler than surgical collection. However, this method of zygote recovery in an embryo transfer program has not previously been reported, presumably because of uncertainties associated with viability of embryos accommodated within the anoxic intra-uterine milieu during the period between stunning of the sow and removal of her uterus on a commercial abattoir slaughter line (about half an hour). In this regard, pig embryos used in the in vitro studies detailed in this Thesis (Chapter 5.2) were collected from slaughtered sows, and the BSA supplemented medium in that experiment proved effective in maintaining about 87% of 4-8 cell pig embryos beyond the blastocyst stage (Table 18). The present experiment examines the efficacy of employing this medium in an embryo transfer program in which embryos are collected from donors following slaughter.

7.3.2 Materials and methods

The donor sow was selected from a group of culled-for-age sows on an intensive piggery at Hamley Bridge, South Australia. The sow was injected i.m. with 1000 i.u. of PMSG at weaning and with 500 i.u. of hCG 72 h later. The sow was on heat the following day and was mated on two consecutive days of standing-heat. The mated animal was slaughtered four days following the first mating.

The flush/transport medium was prepared according to the method detailed in Chapter 5.2.2.

In embryo collection, following removal of the uterus from the slaughtered donor sow (32 min after stunning), 15 ml of warm (37°C) flush/transport medium was flushed from the fimbrial end of each fallopian tube to a point in the uterine body, 20 cm distal to the utero-tubal junction. At this position, a sterile flanged glass tube (90 mm long, 5 mm internal diameter) was inserted through a 5 mm incision in the uterine wall, the flanged end being within the uterine lumen. The flange was broad enough
(about 1 cm) to prevent endometrium from occluding the lumen of the tube. Sterile tape was tied around the uterus to seal the endometrium against the flush tube.

Flush medium was milked through each uterine horn toward the drain tube, and collected directly into sterile, conical base containers (Filtrona, Thomastown, Victoria, Australia). The flushings were transported at 37°C to the NPRU, about 10 km from the abattoir. As a disease precaution, collection bottles were immersed in warm absolute ethanol at the NPRU, then transferred to 37°C. Thirteen ovulation points were observed on the ovaries of the donor sow.

The recipient sow was selected on the basis of cycle synchrony with the donor animal. This gilt was injected with 400 i.u. PMSG on the same day as the donor was treated with PMSG. The PMSG priming routine was implemented to increase the number of CL following ovulation of the recipient which would, in turn, increase the potential for progesterone synthesis and thus benefit the surrogate pregnancy should the natural ovulation rate of the recipient be low. The recipient was not treated with hCG. Twelve morphologically normal 8-cell embryos were recovered from the flushings of the donor sow. Prior to embryo transfer, these embryos were washed twice in flush/transport medium and 6 transferred, in a minimum volume of medium (about 30 ul), to each horn of the recipient uterus, exteriorized by mid-ventral laparotomy. The recipient was not treated with antibiotics post-operatively.

7.3.3 Results

The surrogate pregnancy was confirmed by ultrasound 36 days following transfer, and the recipient gilt subsequently farrowed 109 days following transfer. Ten piglets were born, 2 of which were dead. No signs of abnormality were determined post-natally in any of the liveborn piglets.
7.3.4 Discussion

The pH and osmolarity of flushings of uteri collected from slaughtered sows can be widely different from those of the flushing medium (own unpublished data). In this particular routine, the pH and osmolarity of the flushings were 7.66 and 264 mOsm respectively, and the flushed embryos were in these flushings for about one hour before being washed in clean aliquots of flush/transport medium. Despite the variance between these values and the optimum values of the flush/transport medium (7.40 and 280 mOsm respectively), the success of the ensuing pregnancy indicates that the flushings provided a suitable environment for the flushed embryos and, further, suggests that the MEM/HEPES medium used has potential application in any pig embryo transfer program where delays could occur between collection (abattoir or surgical) and transfer. In this respect, this technique has since been used to transfer embryos between slaughtered donors and on-farm recipients, with delays between collection and transfer of up to 10 hours (pregnancy rate 5/7).

At the time of formal publication of this method (Stone et al., 1984 a), successful transfer of embryos collected from the uterus of a sow slaughtered at a commercial abattoir had not previously been reported. In a practical respect, abattoir collection of embryos holds all the advantages of conventional surgical embryo transfer as a technique for introducing new bloodlines into SPF herds. However, in comparison to surgical flushing techniques, the present method offers additional advantages of simplicity of embryo recovery, facilitating flushing of a large number of uteri at low cost and in a short time.

If this procedure could be coupled to a reliable non-surgical egg transfer technique; collection, transport and transfer of pig embryos would no longer be restricted to those enterprises with surgical facilities. However, as a result of technical complications (Polge & Day, 1968), reliable non-surgical embryo transfer procedures are not presently
available.

There is currently much uncertainty in regard to the risks of transmitting particular diseases by embryo transfer (e.g. Hare, 1984). However, few viruses have been isolated from ova or sperm (Eaglesome et al., 1980) and the zona pellucida is impenetrable to most micro-organisms (ibid). The most probable mechanisms of infection of transferred zygotes are, therefore, contamination of the zona by adhering micro-organisms in the donor uterus, or in the slaughterhouse environment, or by colonization of the transferred embryo by pathogens already present in the host uterus (Singh, Dulac & Hare, 1984). Treatments which are effective in disinfecting zonae, without reducing embryo viability following transfer (e.g. Singh, Hare, Thomas, Eaglesome & Bielanski, 1983), thus provide an extension of the above technique which is consistent with reducing the risk of proliferation of micro-organisms in embryo transfer programs.
GENERAL DISCUSSION
CHAPTER VIII

GENERAL DISCUSSION

Analysis of steroid hormones in uterine washings of sows revealed high levels of pregnenolone sulphate and DHEA sulphate, and indicate that concentrations of these steroid sulphates in histotroph may exceed respective plasma values by up to 160x (Table 5). As pig blastocyst tissues exhibit sulphatase activity (Chapter 5.3; Flint et al., 1979) and show a capacity to synthesize oestrogens from C21 steroids in vitro (Gadsby et al., 1976; Heap et al., 1981), the steroid sulphonyl conjugates accumulated in uterine fluid of sows between Days 9 and 15 after oestrus (Table 4) may provide an important source of precursors to steroidogenesis by preimplantation blastocysts in vivo.

When intact pig blastocysts (Days 3 to 8 after mating) were cultured in media supplemented with authentic pregnenolone sulphate, at concentrations approximating those in histotroph, pregnenolone, progesterone, androstenedione, testosterone, oestradiol and oestrone were released into the surrounding medium (Table 21). Pregnenolone and progesterone were the predominant secretory products, and were found to accumulate in the blastocoele fluid of pig embryos between Days 4 and 8 after fertilization, to concentrations near 4 and 15 μM respectively (Table 20). Preimplantation rabbit blastocysts also accumulate steroids (Seamark & Lutwak-Mann, 1972; Borland, Erickson & Ducibella, 1977) and it has been suggested that release of steroids of conceptus origin (particularly progesterone and oestradiol) may be crucial to the establishment and maintenance of pregnancy (Dickman et al., 1975; Dickman et al., 1976; Dickman, 1979). Published evidence is
consistent with this proposal. For example, oestrogens:

- are potent luteotrophins in the sow (Gardner, First & Casida, 1963; Bazer & Thatcher, 1977; Frank et al., 1978; Ford & Magness, 1980),
- can stimulate endometrial synthesis and release of prostaglandins (Naylor & Poyser, 1975; Geisert, Thatcher & Renegar, 1982) which can mediate the inflammatory reaction and vascular changes at implantation (Kennedy & Armstrong, 1981),
- can stimulate blood flow to the endometrial vascular bed in sows (Dickson, Bosc & Locatelli, 1969; Bazer, Roberts & Sharp, 1978; Ford, Christenson & Ford, 1982),
- are implicated in initiation of blastocyst spacing (Pope, Maurer & Stormshak, 1982) which is critical to embryonic survival in sows,
- stimulate endometrial secretion of protein and calcium (Geisert, et al., 1982 a), and
- are key elements in the recognition of pregnancy in sows (Heap et al., 1977)

Progestins can:

- stimulate release of protein by the endometrium (Murray et al., 1972; Knight, Bazer & Wallace, 1973; Roberts et al., 1976; Schlosnagle et al., 1981; Adams, Bazer & Roberts, 1981),
- activate peptidase and lysozyme activities in the endometrium (Roberts et al., 1976),
- induce inhibition of trypsin activity, which may prevent degradation of conceptus growth factors (Fazleabas, Roberts & Bazer, 1982), and
- act as immunosuppressive agents (Siiteri et al., 1977; Fujisaka et al., 1985) in establishing immune tolerance of embryonic tissues by sows.

In addition, progesterone and oestradiol act synergistically to enhance secretion of macromolecules by endometrial tissues of sows (Knight et al., 1973; Roberts et al., 1976; Basha, Bazer & Roberts, 1980) and of other
species (e.g. mouse - Fishel, 1979).

Maintenance of allogeneic blastocyst tissues as allografts on an immunocompetent endometrial surface (Vaerman & Ferin, 1975), stimulated luteal secretory activity during early pregnancy (Chapter 4.2), enhanced blood flow to endometrial segments in contact with trophoblastic tissue (Ford et al., 1982), and higher secretory activity of endometrial tissues underlying conceptuses (Basha et al., 1980) are, therefore, properties of early pregnancy which are consistent with the present evidence for de novo synthesis and release of progesterone and oestradiol by pig blastocysts in vivo (Chapter 5.3). Alterations in immunosuppression, and other local responses to high steroid concentrations in those areas of the endometrium apposed to trophoblast tissue, may prove to be of special significance to successful implantation in the pig, as its endometrium does not otherwise expose preferred implantation sites (Chapter 2.2). Furthermore, boars are intra-uterine inseminators (Mann, Polge & Rowson, 1956) and, if endometrial immunosuppression was maintained via a systemic route, the spectrum of micro-organisms introduced directly into the uterus at mating (Reed, 1969; Waltz et al., 1968; Thacker, Larsen, Joo & Leman, 1984) could readily colonize that organ and its embryonic contents, and thereby prejudice the success of early pregnancy.

Release of steroids by blastocysts may thus be important in early pregnancy, although increased blood-flow to areas of apposition between embryos and the endometrium (Ford et al., 1982) suggests that local relief from the anti-inflammatory properties of progesterone (Beer & Billingham, 1979) is effected at the implantation sites. Secretion of oestradiol (Table 20) and prostaglandin E2 (Text-figure 16) by blastocysts, as potent endometrial angiogenic hormones (oestradiol- Dickson, Bosc & Locatelli, 1969; Ford, Christenson & Ford, 1982; PGE2- Kennedy, Barbe & Evans, 1980), may effect this relief.

Responses of endometrial tissues to steroid hormones will be dependent upon properties of the local steroid receptors. Steroid receptor activities
in endometrial tissues of early pregnant sows have not yet been examined, but a recent investigation of progesterone and oestradiol receptors in endometrial tissues of the rabbit (Khan-Dawood & Dawood, 1984) has shown synchronized changes in activity of these receptors prior to implantation, with hormonal interaction between preimplantation blastocyst and endometrial tissues promoting implantation without subsequent rejection (ibid).

Studies described in this Thesis have indicated that pregnenolone sulphate is the putative precursor to steroid synthesis by preimplantation blastocyst tissues in pigs. Furthermore, the results suggest that pregnenolone sulphate accumulated in uterine fluids of sows derives from the ovaries, via the maternal circulation. Pregnenolone sulphate is a predominant secretory product of sow ovaries between Days 11 and 17 after oestrus (Tables 13 and 14), and its rate of secretion into the ovarian venous drainage appears to be quantitatively correlated with progesterone release (Text-figure 11). Positive correlations have previously been indicated between embryonic survival and progesterone secretion in early pregnant sows (Glasgow et al., 1951; Mayer et al., 1961), so parallel positive correlations between embryonic survival and pregnenolone sulphate secretion may be assumed. The practical implications of knowledge of this relationship are presently being investigated in this laboratory.

Pregnenolone sulphate is also an abundant component of human uterine fluids (Table 21; Text-figure 15), and of human plasma (Erberlein, 1965; Begue, 1965; Conrad et al., 1967; Sjovall et al., 1968; Scommegna et al., 1971; Peretti & Mappus, 1983). If human embryos are like pig embryos in regard to the capacity to metabolize this steroid sulphonyl conjugate, important roles for pregnenolone sulphate as a component of media used in human IVF/embryo transfer programs may be postulated.

The capacity for pig embryonic tissues to metabolize pregnenolone sulphate (Chapter 5.3), and to convert those metabolites to oestrone (Chapter 5.3; Heap et al., 1975; Gadsby et al., 1976; Perry et al., 1976; Gadsby et al., 1980; Heap et al., 1981) which is sulphonyl conjugated in the
endonretrium and released to the peripheral circulation as oestrone sulphate (Chapter 7.1), provides a basis for quantitating the mass of steroidogenically active embryonic tissue in early pregnant sows (Horne et al., 1983). As most embryos present after the fourth week of gestation survive to term (Text-figure 1), plasma levels of oestrone sulphate during early pregnancy can also be related to numbers of conceptuses later in gestation (Chapter 7.1). This Thesis describes statistically significant correlations between levels of oestrone sulphate in plasma of sows between Days 22 and 29 of gestation, and litter size at term (Table 22). The practical significance of these predictive relationships is discussed (Chapter 7.1).

The inflammatory response which occurs in the endometrium at implantation sites in mammals is characteristic of a hypersensitivity reaction, and the level of the inflammatory response appears to be positively related to the efficiency of implantation and to the size and weight of the resulting foeto-placental unit (literature reviewed in Chapter 7.2.1). Treatment of humans (Taylor & Faulk, 1981), mice (James, 1965), rats (Beer & Billingham, 1974) and hamsters (Beer et al., 1975) to enhance the inflammatory response at implantation sites has thus been shown to stimulate conceptus growth and improve reproductive efficiency. This Thesis reports a significant (P<0.025) increase in litter size of sows immunized by insemination with frozen/thawed boar semen (average litter size of 11.9; compared with a control value of 9.3), plasma levels of oestrone sulphate in the presensitized and control groups being similar (P>0.1). These findings suggest that components of a local inflammatory response by endometrial tissues of sows during early pregnancy can advantage the implanting pig conceptus tissues (e.g. angiogenesis; Chapter 7.2.1).

Improved reproductive efficiency of sows which have been treated to stimulate their immunological response to conceptus tissues at implantation can, however, be viewed as immunological heresy. In this regard, progesterone has immunosuppressive properties (Siiteri et al., 1977), and
Pujisaka et al. (1985) have shown that progesterone and PGE can act synergistically to inhibit the proliferative mitogenic response of T cells in human secretory endometrium. Progesterone and PGE of conceptus origin may thus participate as non-specific immunosuppressive factors in facilitating implantation of histoincompatible conceptus tissues (ibid) and, therefore, the capacity for pig blastocysts to release these hormones (see Chapters 5.3 and 6) may prove critical to the establishment and maintenance of early pregnancy in this species. In addition, treatment of sows and/or their conceptuses to enhance these activities may reduce the high level of early embryonic mortality which is characteristic of early porcine pregnancy (Text-figure 1, Table 1). The findings of this Thesis provide a foundation for more applied investigations on the effectiveness of such treatments (e.g. stimulation of CL secretory function during early pregnancy) in improving reproductive efficiency in sows. Finally, it is hoped that the results of this study provide a broader awareness of the interaction of biochemical activities which, together, comprise the enigma of mammalian implantation.
BIBLIOGRAPHY


BRAMBELL, C.L. (1933) Allantochorionic differentiations of the pig studied morphologically and histochemically. Amer. J. Anat. 52, 397.


BUREAU OF AGRICULTURAL ECONOMICS (1972) Pig raising in Australia - An economic survey.


DZIUK, P.J. (1968) Effect of number of embryos and uterine space on embryo survival in the pig. J. Anim. Sci. 27, 673-676.


POLGE, C. & DZIUK, P.J. (1965) Recovery of immature eggs penetrated by spermatozoa following induced ovulation in the pig. J. Reprod. Fert. 9, 357-358.


ROBSON, J.M. & SHARAF, A.A. (1952) Effect of adrenocorticotropic hormone (ACTH) and cortisone on pregnancy. J. Physiol. 116, 236.


