1

National Library
of Canada

Bibliothèque nationale
du Canada

CANADIAN THESES
ON MICROFICHE

THÈSES CANADIENNES
SUR MICROFICHE

52829

8-315-04935-9

NAME OF AUTHOR/NOM DE L'AUTEUR Jacqueline Anne Carnegie

TITLE OF THESIS/TITRE DE LA THÈSE Studies on the Early Ovine Conceptus: A Combined
Ultrastructural and Histochemical Investigation of the Day 12 to 16 Blastocyst
and the Immunofluorescent Localization of Ovine Chorionic Somatomammotropin in
the Day 14 to 55 Trophoblast.

UNIVERSITY/UNIVERSITÉ Carleton

DEGREE FOR WHICH THESIS WAS PRESENTED/
GRADE POUR LEQUEL CETTE THÈSE FUT PRÉSENTÉE Ph.D.

YEAR THIS DEGREE CONFERRED/ANNÉE D'OBTENTION DE CE GRADE 1980

NAME OF SUPERVISOR/NOM DU DIRECTEUR DE THÈSE Drs. M.E. McCully and H.A. Robertson

Permission is hereby granted to the NATIONAL LIBRARY OF
CANADA to microfilm this thesis and to lend or sell copies
of the film.

The author reserves other publication rights, and neither the
thesis nor extensive extracts from it may be printed or other-
wise reproduced without the author's written permission.

L'autorisation est, par la présente, accordée à la BIBLIOTHÈ-
QUE NATIONALE DU CANADA de microfilmer cette thèse et
de prêter ou de vendre des exemplaires du film.

L'auteur se réserve les autres droits de publication; ni la
thèse ni de longs extraits de celle-ci ne doivent être imprimés
ou autrement reproduits sans l'autorisation écrite de l'auteur.

DATED/DATE December 4, 1980 SIGNED/SIGNÉ J. Carnegie

PERMANENT ADDRESS/RÉSIDENCE FIXE 837 Maryland Avenue 242 CASTLE FRANK RD.

Ottawa, Ontario KANATA, ONTARIO

K2C 0H9

National Library of Canada
Collections Development Branch

Canadian Theses on
Microfiche Service

Bibliothèque nationale du Canada
Direction du développement des collections

Service des thèses canadiennes
sur microfiche

NOTICE

The quality of this microfiche is heavily dependent upon the quality of the original thesis submitted for microfilming. Every effort has been made to ensure the highest quality of reproduction possible.

If pages are missing, contact the university which granted the degree.

Some pages may have indistinct print especially if the original pages were typed with a poor typewriter ribbon or if the university sent us a poor photocopy.

Previously copyrighted materials (journal articles, published tests, etc.) are not filmed.

Reproduction in full or in part of this film is governed by the Canadian Copyright Act, R.S.C. 1970, c. C-30. Please read the authorization forms which accompany this thesis.

**THIS DISSERTATION
HAS BEEN MICROFILMED
EXACTLY AS RECEIVED**

AVIS

La qualité de cette microfiche dépend grandement de la qualité de la thèse soumise au microfilmage. Nous avons tout fait pour assurer une qualité supérieure de reproduction.

S'il manque des pages, veuillez communiquer avec l'université qui a conféré le grade.

La qualité d'impression de certaines pages peut laisser à désirer, surtout si les pages originales ont été dactylographiées à l'aide d'un ruban usé ou si l'université nous a fait parvenir une photocopie de mauvaise qualité.

Les documents qui font déjà l'objet d'un droit d'auteur (articles de revue, examens publiés, etc.) ne sont pas microfilmés.

La reproduction, même partielle, de ce microfilm est soumise à la Loi canadienne sur le droit d'auteur, SRC 1970, c. C-30. Veuillez prendre connaissance des formules d'autorisation qui accompagnent cette thèse.

**LA THÈSE A ÉTÉ
MICROFILMÉE TELLE QUE
NOUS L'AVONS REÇUE**

STUDIES ON THE EARLY OVINE CONCEPTUS.
A COMBINED ULTRASTRUCTURAL AND HISTOCHEMICAL INVESTIGATION
OF THE DAY 12 TO 16 BLASTOCYST AND
THE IMMUNOFLOUORESCENT LOCALIZATION OF OVINE CHORIONIC
SOMATOMAMMOTROPIN IN THE DAY 14 TO 55 TROPHOBLAST

by

JACQUELINE ANNE CARNEGIE, B.Sc., M.Sc.

A thesis submitted to the Faculty of
Graduate Studies and Research in partial fulfilment
of the requirements for the degree of
Doctor of Philosophy

Department of Biology
Carleton University
Ottawa, Ontario.
August 13, 1980.

The undersigned hereby recommend to the Faculty of
Graduate Studies and Research acceptance of this thesis, submitted by
Jacqueline Anne Carnegie, B.Sc., M.Sc., in partial fulfilment of the
requirements for the degree of Doctor of Philosophy.

Graythorn
.....
Chairman, Department of Biology

M.E.M. Cully
.....
Supervisor

Harold A. Ashton
.....
Supervisor

A.C. Enders
.....
External Examiner

Carleton University

August 13, 1980

Abstract

This research project consists of 2 parts. The first is a correlated investigation of the ultrastructure and histochemistry of the outer embryonic membrane of the pre-attachment sheep blastocyst. It is followed by the localization, using immunofluorescent techniques, of a protein in this embryonic membrane using an antibody to ovine chorionic somatomammotropin (oCS).

The period day 12 to 16 of gestation is a time of rapid expansion of the blastocyst prior to the establishment of placental contact with the mother. At this time the outer embryonic membrane, or trophoblast, is composed of a single layer of cells with a rich coating of microvilli extending out toward the uterine epithelium. The trophoblastic cells, which are filled with polysomes and granular endoplasmic reticulum, are joined firmly to each other by rows of desmosomes along their adjacent boundaries. The appearance of increasing numbers of osmophilic droplets in these cells which stain positive with sudan black B indicates that they are accumulating lipid over this period. Numerous lysosomal-like organelles, their digestive role verified by the demonstration of acid phosphatase activity in glycol methacrylate-embedded (GMA) sections, populate the trophoblast over this interval. The lysosomal constituents, in addition to cellular debris such as membrane fragments and clumps of chromatin-like material, vary with the stage of gestation and range from 0.1 μ m bodies with electron-dense cores at day 12 to large,

protein-containing crystals at day 16. It is postulated that the lysosomes perform 2 roles, the digestion of uterine secretions released to nourish the developing blastocyst and the breakdown of certain of the trophoblastic cells which, for some as yet undetermined reason, die and are phagocytised by their neighbours. These dying cells, identified by a loss of the plasma membrane and, hence, much of the cytoplasmic ground substance, appear to be randomly interspersed among the healthy trophoblastic cells throughout the interval day 12 to 16. They are a normal feature of ovine trophoblastic development and it is suggested that they may arise either as a result of genetic defects in cells which render them incapable of further differentiation and survival or to facilitate the shaping of the trophoblastic membrane as a whole. The development of binucleate cells, beginning at day 16, is described.

A hormone, postulated to be oCS, was localized in sections of GMA-embedded trophoblastic tissue between days 14 and 55 of gestation using Coon's double antibody technique. No hormone was found to be present at day 12; peak levels were observed at day 28 with lesser amounts at days 14 and 55. Positive staining, using rabbit anti-oCS followed by fluorescein isothiocyanate (FITC)-labelled goat anti-rabbit γ globulin, is confined entirely to the cytoplasm of uninucleate trophoblastic cells, in close proximity to lipid droplets. Finally, a discussion of the current status of immunolocalization techniques, especially regarding antiserum specificity, is presented in an appendix.

Acknowledgements

I would like to express my sincere thanks to Drs. M. E. McCully, Department of Biology, Carleton University and H. A. Robertson, Animal Research Institute, Ottawa, who gave much advice and guidance throughout the course of this research. I am indebted also to Janet Clarke, Department of Biology, Carleton University for much valuable advice in performing some of the microscopic and photographic techniques.

I am grateful to Dr. R. S. Gowe, Director, Animal Research Institute, for his permission to use the animal facilities of the Animal Research Institute for some of this research. I am also grateful to the National Research Council of Canada and Gulf, Canada Ltd. who provided financial support during the years required to complete this research.

I would like to thank Drs. J. S. D. Chan and H. G. Friesen for the antiserum to oCS (ovine chorionic somatomammotropin) used in the immunolocalization studies. I would also like to thank John Shackleton, Animal Research Institute, Ottawa, who supplied the photographs for Figures 1 and 2.

I would like to thank my husband, Jim Carnegie, for his patience and understanding during my years as a PhD student.

Table of Contents

	Page
Abstract	iii
Acknowledgements	v
List of Tables	viii
List of Diagrams	ix
List of Figures	x
Part 1. A Combined Ultrastructural and Histochemical Study of the Sheep Trophoblast (<u>Ovis aries</u>) from Days 12 to 16 of Gestation.	
Introduction	1
Materials and Methods	14
1) Collection and Processing of Tissue	
2) Sectioning and Staining	
3) Histochemistry	
a) Tissue embedded in GMA	
b) Tissue embedded in Spurr's resin	
4) Microscopy and Photomicrography	
Results	19
Discussion	45
Part 2. The Immunofluorescent Localization of Ovine Chorionic Somatomammotropin in the Days 14 to 55 Ovine Trophoblast.	
Introduction	61
Materials and Methods	70
1) Collection and Handling of Tissue	
2) Tissue Sectioning and Incubation	
3) Preincubation Control	

	<u>Page</u>
Results	74
Discussion	82
Appendix A. Solutions and Methods used in Tissue Preservation and Staining	95
Appendix B. Immunofluorescent Techniques - A Critical Appraisal	102
Appendix C. Carnegie, J.A., McCully M.E. and H.A. Robertson, 1980: Embedment in glycol methacrylate at low temperature allows immuno- fluorescent localization of a labile tissue protein. J. Histochem. Cytochem. <u>28</u> , 308-310	
References	114

List of Tables

<u>Table No.</u>		<u>Page</u>
1	Summary of the developmental events in the early life of the ovine conceptus.	2
2	Blastocyst growth between days 10 and 14 of gestation. Data from 2 separate studies.	9
3	Summary of tissues incubated for oCS.	72
4	Summary of data - Immunolocalization of oCS.	83-84
5	Summary of data - Immunolocalization of hCS.	86-87

List of Diagrams

<u>Diagram No.</u>		<u>Page</u>
1	Blastocyst at about day 9 of gestation.	7.
2	Blastocyst at about day 12 of gestation.	7
3	A comparison of sensitivity using single and double antibody methods.	103
4	A comparison of antiserum specificity in radioimmunoassay and immunolocalization.	106

List of Figures

<u>Figure No.</u>		<u>Page</u>
1	Nonpregnant ovine reproductive tract, opened to show the caruncles.	3
2	Several caruncles shown at higher magnification.	3
3	Sketch showing a cross section through fetal trophoblast and maternal caruncle at roughly day 16, as placental development is about to begin.	3
4	Sketches of blastocysts at days 12 and 16, to scale, indicating tremendous growth of the trophoblast over this interval.	5
5	Entire day 12 blastocyst.	5
6	Junction of fetal trophoblast and maternal caruncle at day 16.	5
7	Sketch of a typical day 12 blastocyst.	20
8	Portion of typical day 12 trophoblast.	20
9	Day 14 trophoblastic cell.	20
10	Region of cytoplasm taken from a day 14 trophoblastic cell.	20
11	Junction between two day 16 trophoblastic cells.	21
12	Enlargement of indicated region in Fig. 11 showing fine structure of a single desmosome.	21
13	A desmosome at a junction between the slender cytoplasmic processes of 2 endodermal cells.	21
14	A typical day 12 trophoblastic cell, showing the absence of cytoplasmic lipid droplets.	23

<u>Figure No.</u>		<u>Page</u>
15	Portion of a day 14 trophoblastic cell; several lipid droplets are present in the cytoplasm.	23
16	Day 16 trophoblastic cell showing the presence of numerous lipid droplets in the cytoplasm.	23
17	Day 14 trophoblast stained with sudan black B.	24
18	Day 16 trophoblast stained with sudan black B.	24
19	Another region of day 16 trophoblast treated in the same manner and giving the same information as that seen in Fig. 18.	24
20	Portion of trophoblastic-caruncular junction, again stained with sudan black B.	24
21	Portion of day 12 trophoblastic cell showing several typical lysosomes.	26
22	Day 14 lysosome in which a single crystal of the size and shape always encountered at this stage is seen.	26
23	Day 16 trophoblastic cell containing 2 lysosomes.	26
24	Acid phosphatase reaction on day 14 trophoblastic tissue shown in colour.	27
25	Day 14 trophoblastic tissue incubated to show localization of acid phosphatase activity.	27
26	Day 12 trophoblastic tissue incubated to show localization of acid phosphatase activity.	27
27	Electron micrograph of a day 14 trophoblastic cell previously incubated for acid phosphatase for optical microscopy.	27

<u>Figure No.</u>		<u>Page</u>
28	Lysosomes in a day 12 trophoblastic cell which contain numerous 0.1 μ m bodies.	28
29	A single day 12 lysosome containing cellular debris and many 0.1 μ m bodies.	28
30	Small bodies in the microvillar region of a day 12 trophoblastic cell.	28
31	Two 0.1 μ m bodies in the microvillar region of a day 12 trophoblastic cell shown at high magnification.	28
32	Day 12 lysosomes containing 0.1 μ m bodies.	28
33	Day 14 trophoblastic cell containing a lysosome in which are located three 0.1 μ m bodies and a crystal, along with considerable flocculent material.	30
34	Small lysosome in day 16 trophoblastic cell containing a few 0.1 μ m bodies.	30
35	Day 16 trophoblastic lysosome containing 0.1 μ m bodies.	30
36	Several small lysosomes in a day 16 trophoblastic cell, two of which contain recognizable 0.1 μ m bodies.	30
37	Day 14 lysosome containing a single crystal along with considerable cellular debris.	31
38	Day 16 lysosome containing many crystals of various sizes and a small amount of cellular debris.	31
39	Cluster of crystals within a day 16 trophoblastic cell.	31
40	Cross section of the crystal in Fig. 39 shown at higher magnification.	31
41	Portion of a crystal shown in grazing cross section.	31

Figure No.

Page

42	A group of crystals in a day 16 trophoblastic cell around which a recognizable lysosomal membrane could not be discerned.	32
43	Small cluster of crystals in a maternal caruncular epithelial cell at day 16 of gestation.	32
44	A single crystal in day 18 maternal caruncular epithelium which is closely bounded by a single membrane.	32
45	Three crystals in the middle to basal region of a day 16 maternal caruncular epithelial cell.	32
46	A small, membrane-bounded bundle of crystals in a maternal caruncular epithelial cell at day 22 of gestation.	32
47	Section of day 30 trophoblastic-caruncular junction digested with papain and stained with aniline blue black.	34
48	Control section of day 30 trophoblastic-caruncular junction incubated with buffer lacking papain and stained with aniline blue black.	34
49	Section of day 30 trophoblastic-caruncular junction stained with naphthol yellow S.	34
50	Section of day 28 trophoblastic-caruncular junction stained with mercury-bromphenol blue.	34
51	Section of day 30 trophoblastic-caruncular junction stained with aniline blue black.	34
52	One of the few differentiating binucleate cells observed in day 16 trophoblastic tissue.	35
53	Day 16 trophoblastic cell which appears to be in the process of becoming a binucleate cell.	35

<u>Figure No.</u>		<u>Page</u>
54	Part of the junction between the possible differentiating binucleate cell in Fig. 53 and its neighbouring uninucleate cell.	35
55	Day 18 trophoblastic binucleate cell containing a crystal.	35
56	Day 22 trophoblastic binucleate cell containing extensive Golgi complex basally and possible secretory granules apically.	36
57	Day 16 trophoblastic-caruncular junction stained with PAS.	36
58	Day 30 trophoblastic-caruncular junction stained with PAS.	36
59	Portion of day 16 trophoblastic tissue showing a pale-staining cell in the process of disintegrating and a second fairly pale-staining cell which appears to have been engulfed by another trophoblastic cell.	39
60	Portion of day 12 trophoblastic tissue in which a pale-staining disintegrating cell is located adjacent to an endodermal cell undergoing replication.	39
61	Cluster of dying cells located in a portion of day 14 trophoblastic tissue.	39
62	Day 12 trophoblastic cell in the process of dying.	40
63	Junction between a dying day 12 trophoblastic cell and one that is apparently healthy.	40
64	A disintegrating day 16 trophoblastic cell with a healthy neighbouring cell.	40
65	Near-adjacent section to the one used for Fig. 64. It can be seen that the dying cell is only one in a series of healthy trophoblastic cells.	40

<u>Figure No.</u>		<u>Page</u>
66	Nucleus in a day 16 dying trophoblastic cell.	40
67	Serial sections showing that a dying cell in day 16 trophoblastic tissue is adjacent to a healthy cell.	42
68	Serial sections through day 16 trophoblastic tissue.	43
69	Day 28 trophoblast corresponding to that in Fig. 70 - phase contrast optics.	75
70	Binding of anti-oCS to day 28 trophoblastic tissue.	75
71	Binding of anti-oCS to day 28 trophoblastic tissue.	75
72	Corresponding area to that in Fig. 71 - phase contrast optics.	75
73	Binding of anti-oCS to the cytoplasm of day 14 trophoblastic tissue.	75
74	Binding of anti-oCS to the cytoplasm of a day 14 trophoblastic cell.	76
75	Portion of day 16 trophoblast showing positive staining for oCS in most of the cells.	77
76	Day 28 trophoblastic tissue with positive staining for oCS in the cytoplasm of uninucleate cells, but not in 2 binucleate cells.	77
77	Corresponding region to that in Fig. 76 - phase contrast optics.	77
78	Section of day 28 placental tissue showing the junction between fetal trophoblast and maternal caruncular tissues.	79
79	One of the few examples of positive staining for oCS toward the crypts between the maternal villi.	79

<u>Figure No.</u>		<u>Page</u>
80,	Same area as that in Fig. 79 - phase contrast optics.	79
81	Day 55 caruncular trophoblast incubated for oCS.	80
82	Same region as that shown in Fig. 81 - phase contrast optics.	80
83	Day 55 caruncular trophoblast incubated for oCS.	80
84	Same region as that shown in Fig. 83 - phase contrast optics.	80

Part 1. A Combined Ultrastructural and Histochemical Study of the
Sheep Trophoblast (*Ovis aries*) from Days 12 to 16 of Gestation.

Introduction.

At the time of its release by the ovary, the ovine egg is a single cell surrounded by a mucopolysaccharide-containing coat about 0.014 mm thick (Clark, 1934) termed the zona pellucida. Its total diameter 4.5 h after mating is 0.146 mm and, in fact, an average of diameters taken from this time until 138.75 h post coitum (5.75 days p.c.) of 0.147 mm (range is 0.135 - 0.160 mm) indicates that it does not begin to enlarge appreciably until after the zona pellucida has been shed at roughly 7 days p.c. (Clark, 1934). Fertilization occurs in the ampulla of middle region of the oviduct (Fig. 1) (Davies and Hesseldahl, 1971) and by 38 - 39 h p.c. the ovum has reached the 2-cell stage (Clark, 1934; Green and Winters, 1945; McLaren, 1972). The developmental sequence described in the next few pages is summarized in Table 1. The ovum spends 2 to 4 days moving down the oviduct and, during this time, the uterine environment changes from one favouring spermatozoan survival to one supportive of the further development and, ultimately, the implantation of the conceptus (McLaren, 1972). Successive muscular contractions under the control of the steroid hormones estrogen and progesterone help the zygote to move down the oviduct (Davies and Hesseldahl, 1971) and the suggestion has been made that one function of the zona pellucida is to keep this loosely-bound cluster of dividing cells

Table 1. Summary of the developmental events in the early life of the ovine conceptus.

Time post coitum	Event	References
38-39 h	Cleavage of fertilized egg to produce 2 blastomeres	6,9,10
4 days	Reaches uterus between 16- and 32-cell stages	6
4.5-5 days	Becomes a morula	5,11
5.5-6 days	Develops into a blastocyst	5,11
7-8 days	Sheds zona pellucida	11
7-11 days	Blastocyst acquires a complete lining of endodermal cells	2,4,9,12
10-15 days	Blastocyst elongates rapidly from roughly 1 mm to 70-100 mm	1,11
12-13 days	Amniotic folds first appear	4,8,9
13-15 days	Embryo passes through primitive streak stage and develops notochord and neural plate. Becomes enclosed by amniotic sac.	4,8,9
15 days	Allantoic sac begins to develop.	4,9
16-18 days	Implantation begins? Embryo has about 20 somites, a heart bulge and some of the major blood vessels.	1,2,3,4,6 4

1. Bindon, 1971	7. Clark, 1934
2. Boyd and Hamilton, 1952	8. Evans and Sack, 1973
3. Boshier, 1969	9. Green and Winters, 1945
4. Bryden <u>et al.</u> , 1972	10. McLaren, 1972
5. Calarco and McLaren, 1976	11. Rowson and Moor, 1966
6. Chang and Rowson, 1965	12. Wintenberger-Torrès and Fléchon, 1974

FIGURE 1

Nonpregnant ovine reproductive tract; the uterine horns have been opened to show the caruncles (Car), the sites of placental attachment. Ov = ovary; Ovid = oviduct. Photograph courtesy of John Shackleton, Animal Research Institute, Ottawa.

FIGURE 2

Several caruncles (Car) from Fig. 1 are shown at higher magnification. Photograph courtesy of John Shackleton, Animal Research Institute, Ottawa.

FIGURE 3

Sketch showing a cross section through fetal trophoblast (Tr) and maternal caruncle (Car) at roughly day 16, as placental development is about to begin. E = endodermal cell; ME = maternal epithelial lining of caruncle.

intact and from adhering to the oviduct walls during its journey (McLaren, 1972).

The conceptus reaches the uterus during day 4 at some point between the 16- and 32-cell stages (Clark, 1934). It becomes a morula at roughly day 4.5 - 5 and a blastocyst within the next 24 h or so (Rowson and Moor, 1966; Calarco and McLaren, 1976). The blastocyst (Figs. 5 and 7) is a fluid-filled vesicle whose wall is composed of a single layer of trophoblastic cells and which contains, at one pole, a cluster of rather undifferentiated cells, the inner cell mass (ICM), which will give rise to the embryo proper plus its associated membranes, the amnion, allantois and yolk sac (Davies and Hesseldahl, 1971). The trophoblast, on the other hand, will develop into the chorion, the membrane responsible for establishing placental contact with the mother. This is accomplished by the successive migration of 2 cell layers from the ICM (Diagram 2); first, an inner lining of endodermal cells and then a middle layer of mesodermal cells with associated fetal vascular elements (Boyd and Hamilton, 1952). Once the trophoblast has acquired a blood supply, it is called a chorion and has become capable of exchanging nutrients, oxygen and waste material with the mother.

The conceptus retains the zona pellucida up to early blastocyst formation. It has been determined through observations of 190 blastocysts during the interval 128 - 224 h (5.33 - 9.33 days) post estrum that this protective coat is shed about halfway through this period, following which the blastocyst becomes somewhat enlarged (Rowson and Moor, 1966).

FIGURE 4

Sketches of blastocysts at days 12 and 16, to scale, indicating the tremendous growth of the trophoblast over this interval.

FIGURE 5

Portion of day 12 blastocyst. Photograph was taken of block face from which some cross sections had already been removed. ICM = inner cell mass; Tr = trophoblast.

FIGURE 6

Junction of fetal trophoblast (FTr) and maternal caruncle at day 16; orientation corresponds to that in Fig. 3. A few endodermal cells (E) can be seen in the upper part of the micrograph. c = crystal; ME = epithelial lining of maternal caruncle; mv = interdigitating microvilli of fetal and maternal layers. Paragon stain; brightfield optics.

④

trophoblast

2 cm

⑤

0.2 mm

⑤

ICM

Tr

At the time of its initial formation, the blastocyst consists of a complete sphere of flattened trophoblastic cells encapsulating both the blastocoel fluid and the ICM (Diagram 1). By days 9 or 10, the embryonic cells are no longer enclosed by the trophoblast, but have themselves become a part of this outer layer (Bryden et al, 1972). Studies using scanning electron microscopy (Fléchon, 1978) have shown that holes begin to appear in the ovine trophoblastic layer at about day 10. By days 11 to 12, these have merged to become one large cavity (200 - 250 μ m in diameter), irregular in outline, through which a prominent bulge of embryonic cells protrudes (Fléchon, 1978). As the ICM intercalates into the trophoblast, it develops from a loosely organized, roughly spherical cluster of cells into a flattened embryonic disc which, by day 12, extends above the trophoblastic boundary (Diagram 2) (Boyd and Hamilton, 1952; Bryden et al, 1972). Coincident with formation of the embryonic disc is the migration of endodermal cells from this source to form a complete inner lining to the trophoblast by days 9 to 11 (Green and Winters, 1945; Boyd and Hamilton, 1952; Bryden et al, 1972; Wintenberger-Torrès and Fléchon, 1974).

The amniotic folds first appear at days 12 or 13 (Green and Winters, 1945; Bryden et al, 1972; Evans and Sack, 1973) and within the next 2 days grow up to enclose the embryo in a sac (Bryden et al, 1972; Evans and Sack, 1973). During this interval, the embryo passes through the primitive streak stage and the notochord and neural plate are formed (Green and Winters, 1945; Bryden et al, 1972). The allantois, that vesicle which will

Diagram 1. Blastocyst at about day 9 of gestation

Diagram 2. Blastocyst at about day 12 of gestation

collect embryonic wastes, first begins to develop at about day 15 (Green and Winters, 1945; Bryden et al, 1972) and contains fluid by the 18th day (Davies, 1952).

At days 10 and 11 of gestation, the blastocyst is spherical to ovoid and measures 1 mm or less in diameter (Bindon, 1971). Over the next 4 days it elongates extremely rapidly and by the end of this period can measure from about 70 mm (Bindon, 1971) to over 100 mm (Rowson and Moor, 1966) in length. Table 2 and Fig. 4 give some idea of the rapid rate of growth of the trophoblast at this time. From this tabulation of data, it is apparent that the assignment of expected blastocyst lengths to days of gestation over this interval of rapid elongation cannot be done for the following two reasons. First, the lengths change dramatically within hours and, second, the timing of gestation from the day of estrus necessitates an error of at least 12 h in relating stage of gestation to time of ovulation. However, one can say that during the period between days 10 and 15 post estrum the blastocyst becomes extremely long without changing appreciably in width and that by the time attachment to the uterine endometrium commences, it extends throughout most of both uterine horns.

Beginning at roughly day 10 of gestation, the conceptus is held loosely in place against the uterine wall by a sticky material coating its outer surface (Green and Winters, 1945). For several days no structural developments are made to strengthen this adhesion and as late as day 16 entire conceptuses are easily removed from the uterine horns simply by flushing with buffered

Table 2. Blastocyst growth between days 10 and 14 of gestation.
Data from 2 separate studies.

a) Bindon, 1971

Day of gestation*	No. of blastocysts	Length of trophoblast (mm)	Width of trophoblast (mm)
10	4	0.43 ± 0.09	0.34 ± 0.09
12	5	32.9 ± 13.1	1.01 ± 0.21
14	7	67.9 ± 17.2	1.14 ± 0.38

*In this study, original data reported with day 1 = day of estrus. This was adjusted to day 0 = day of estrus for easier comparison with that reported by Rowson and Moor, 1966.

b) Rowson and Moor, 1966.

Data for days 12 to 14 taken from 123 undamaged blastocysts.

Day of gestation*	Length of trophoblast (mm)**
11	1
12	11.7
13	35.1
14	102.3

*Day 0 = day of onset of estrus

**In the presentation of their data, the standard deviation was shown on the graph, but not included in the table.

saline (Carnegie; unpublished observation). This initial adhesion of the conceptus to the uterine lining should not be confused with the actual attachment process which, for the purposes of this discussion, is defined as the formation of interlocking villi between maternal and fetal placental tissues and which, according to most of the evidence in the literature, commences at some point between days 16 and 18 (Boyd and Hamilton, 1952, Chang and Rowson, 1965; Boshier, 1969; Bindon, 1971; Bryden et al, 1972). At this time the embryo itself has about 20 somites and has developed a heart bulge and some of the larger blood vessels (Bryden et al, 1972).

An understanding of the process of attachment requires some knowledge of the structure of the ovine uterus. If the uterus of a pregnant or nonpregnant sheep were opened, one would see numerous button-like projections dotting the endometrium (Figs. 1 and 2). Each uterine horn contains roughly 45 to 50 of these caruncles and they are the site of placental formation in ruminants such as the sheep and the cow (Harvey, 1959). Following blastocyst expansion, the trophoblast comes to lie over the caruncles (Figs. 3 and 6) and at each region of apposition tremendous vascular development occurs in both the fetal (cotyledon) and maternal (caruncle) tissues. Interdigitating villi bring the two circulatory systems into close proximity and the resultant structure, the site of gaseous, nutrient and waste exchange between the fetus and mother, is called a placentome. By day 16, the stage at which this study was concluded, the blastocyst has sufficiently expanded to permit fetal trophoblast and maternal

caruncle to come into contact and placentome formation is about to begin (Fig. 6).

The fine structure of the developing ovine blastocyst has already been investigated to some extent. Calarco and McLaren (1976) studied 14 embryos collected between the zygote and early blastocyst stages (0.5 to 6.5 days after mating) and described numerous ultrastructural features indicating that development, at this time, does not differ appreciably from that of other mammalian species. Wintenberger-Torrès and Fléchon (1974) looked at blastocysts recovered from 6 ewes between days 8 and 18 of gestation. This is the period of tremendous blastocyst elongation and the initiation of placental formation. It is a critical stage in the life of the embryo and is characterized by the development of many new cell features in the trophoblast, some of which may be peculiar to ovine embryos alone. While the paper by Wintenberger-Torrès and Fléchon (1974) did report many of these developmental changes, the small number of animals studied (1 animal at each of days 8, 10, 12, 14, 16 and 18), the low resolution of the electron micrographs and the lack of accompaniment of the ultrastructural observations with histochemical verification have rendered further study of the critical period in blastocyst development necessary.

Some examples of their observations requiring further study can be given. They reported the presence of trophoblastic cells differing in staining intensity (they were divided into 2 groups, either "dark" or "clear" cells) in days 8 and 10 blastocysts. While some description of the former was given, none at all was

provided for the latter and the 2 electron micrographs of portions of "light" cells provided in the paper are not of high enough magnification to indicate what ultrastructural features are responsible for the different staining characteristics of these 2 groups of cells. Furthermore, no mention is made of the presence of cell types with different staining properties in days 12 to 18 blastocysts and one is left wondering about their fate subsequent to day 10 and, indeed, if they are routinely found in day 8 and day 10 blastocysts.

Wintenberger-Torrès and Fléchon (1974) observed small bodies roughly $0.1\mu\text{m}$ in diameter in the day 12 blastocyst whose role at this stage of gestation they were unable to discern. They were seen only at day 12 and were present in both the microvillar region and in cytoplasmic vesicles of the trophoblastic cells. Two questions immediately come to mind. If more day 12 blastocysts were studied at the ultrastructural level, would these "corpuscles" be seen and, secondly, are they present for only the brief period indicated by Wintenberger-Torrès and Fléchon (1974) or can they be seen in blastocysts collected earlier or later than day 12?

Finally, this combined ultrastructural and histochemical investigation was initiated because insufficient information is available in the literature for the interpretation of the experimental results of the other part of the research project, that is, the localization of the protein hormone ovine chorionic somatomammotropin (oCS) in early trophoblastic tissue by immunofluorescence. At the time of its initial detection (day 14),

oCS is present in every trophoblastic cell; whereas by day 28, its distribution has become quite localized and is confined to certain uninucleate trophoblastic cells which are often clustered in small groups. A goal of the ultrastructural study was to follow the differentiation of the trophoblast into different cellular types and to determine those structural features which characterize the oCS-containing cells.

Materials and Methods

1. Collection and Processing of Tissue

The estrous cycles of the ewes used in this research project and that involving the localization of oCS were synchronized using fluorogestone acetate (FGA) and pregnant mares serum gonadotrophin (PMSG). The advantage of this procedure is that it allows the time of estrus to be controlled and known so that the stage of gestation can be related to the time of ovulation plus or minus 12 h. Intravaginal sponges containing 40 mg FGA (Chrono-gest. Intervet., Angers, France, 49002) were inserted and left for 12 days. They were removed at 1600 h on day 12 and each animal immediately injected intramuscularly with 450 IU PMSG (Ayerst Laboratories). Estrus and hence breeding began 36 h later.

Eight cross-bred ewes were sacrificed between days 12 and 16 of gestation (three at each of days 12 and 14 and one at each of days 15 and 16) and the uteri immediately removed and their contents obtained by flushing with phosphate buffered saline, pH 7.4 (Appendix A). Using a pasteur pipet, each day 12 conceptus was transferred intact to ice-cold 3% glutaraldehyde (Polysciences) in 0.1 M Sorenson's buffer, pH 6.8 (Appendix A) while, at the 2 later stages, pieces of trophoblast 1.5 - 2 mm long were cut and placed in the fixative. Tissue destined for ultrastructural study was fixed 0.5 h, rinsed in buffer and immediately dehydrated and embedded in the water-soluble plastic

glycol methacrylate (GMA) at low temperature (Carnegie et al, 1980, see Appendix C). Two additional animals were included for comparison in the histochemical study, one at day 28 and one at day 30 of gestation.

A second animal was sacrificed at day 16 and one at each of days 18 and 22 and, immediately after removal of the uterus, about half the allantoic fluid was replaced with cold 3% glutaraldehyde using a syringe. This was left for 0.5 h to fix the trophoblast in place against the maternal uterine epithelium. The uterus was then opened and the entire caruncles with the overlying trophoblast carefully excised and fixed a further 2 h in 3% glutaraldehyde to ensure tight adhesion of the trophoblast to the uterine tissue throughout the subsequent treatments. Small pieces, about 1 mm X 1 mm X 2 mm were taken at the junction of the fetal trophoblast and the maternal uterine epithelium, fixed an additional 2 h in fresh, ice-cold 3% glutaraldehyde and rinsed several times in buffer. Tissues for the ultrastructural study were postfixed 1 h in 1% (Sorenson's buffer) osmium tetroxide on ice, dehydrated through an ice-cold alcohol series (5%, 10%, 20%, 40%, 60%, 80%, 90%, 95% and 3 changes of 100%) and infiltrated and flat-embedded in Spurr's resin (Appendix A).

2. Sectioning and Staining

Silver sections were cut using an LKB ultramicrotome and stained sequentially in 2% aqueous uranyl acetate and Reynold's lead citrate (Appendix A). Alternating with each grid of silver sections, thicker sections, showing gold to purple interference

colours, were cut, transferred onto glass slides and stained with Paragon stain (Appendix A) for comparative morphological study with the optical microscope.

3. Histochemistry

a) Tissue embedded in GMA

Using a Porter Blum MT-2 ultramicrotome, 1 - 2 μ m sections were cut with a glass knife, transferred to drops of water on glass slides and air dried at room temperature. All histochemical incubations could be carried out directly on the sections without prior removal of the plastic (Feder and o'Brien, 1968).

Grids of thin sections were also prepared from GMA-embedded day 14 trophoblast and used for the localization of acid phosphatase activity at the electron microscope level. The sections were cut dry using a glass knife equipped with a boat. The boat was then filled with sterile distilled water and suitable sections, displaying pink to yellow interference colours, which had floated out on the surface of the water, were quickly picked up on formvar-coated nickel grids.

The demonstration of acid phosphatase activity was accomplished using the method of Barka and Anderson (1962) with one slight modification (see Appendix A). Control slides in which the substrate was omitted or the enzyme inactivated by immersion of the sections in boiling water prior to incubation (Ashford and McCully, 1970) always yielded negative results.

The same incubation procedure was carried out on the grids of GMA-embedded tissue which were mounted for light microscopy

by suspension in a drop of water hanging from a coverslip inverted over a ring of plasticene placed on a second, larger coverslip and areas of acid phosphatase activity were photographed. The grids were subsequently stained for 5 min each in 2% aqueous uranyl acetate and lead citrate and electron micrographs taken of the same tissue regions.

Three histochemical reactions, naphthol yellow S (Deitch, 1955), mercury-bromphenol blue (Barka and Anderson, 1965) and aniline blue black (Fisher, 1968) were used to test for the presence of protein (Appendix A). In some instances, they were combined with pretreatment of the sections with one of two proteolytic enzymes, protease or papain (Appendix A).

Days 16 and 30 tissue was incubated in the periodic acid-Schiff's (PAS) reaction (Appendix A).

b) Tissue embedded in Spurr's resin

Tissue was tested for the presence of lipid by staining sections from which the osmium tetroxide had been removed using hydrogen peroxide with sudan black B (Appendix A).

4. Microscopy and Photomicrography

The thin sections were examined in a Siemens 1A electron microscope and the information recorded on Kodak electron image glass plates. Optical microscopy was with a Carl Zeiss microscope. In the case of those sections incubated for acid phosphatase or the mercury-bromphenol blue or naphthol yellow S reactions, phase contrast optics were used to enhance tissue detail and photo-

micrographs were recorded on 4 X 5" Kodak high contrast pan sheet film. Kodak 4 X 5" Ektapan sheet film was used to record all other black and white photomicrographs. The acid phosphatase reaction was recorded in colour using Kodak Kodachrome 25 film.

Results

A cross section through the centre of a day 12 blastocyst reveals a ring of squamous to cuboidal-shaped trophoblastic cells lined by a layer of flattened endodermal cells with long, thread-like cytoplasmic processes (Figs. 7 and 8). The embryonic cells (inner cell mass), clustered at one pole, bulge slightly above the adjacent trophoblast (Fig. 7). Numerous prominent microvilli extend outward from the trophoblast toward the uterine wall (Figs. 7 to 9). These are especially apparent at days 12 and 14 (Figs. 8 and 9) but have become somewhat less conspicuous by day 16 (Fig. 16). Over the 5 day period no trend toward an overall increase or decrease in trophoblastic cell size is indicated; cell heights and widths up to 15 μ m were observed at all stages. However, they do change somewhat in shape, being predominantly flattened at day 12, cuboidal at day 14 and cuboidal or columnar at days 15 and 16.

Throughout this interval the cytoplasm of the trophoblastic cells is rich in ribosomal clusters or polysomes and in granular endoplasmic reticulum (Figs. 10 and 11). Often, this granular endoplasmic reticulum (ER) is somewhat distended (Fig. 10, arrows).

Between days 12 and 16, desmosomes are abundantly distributed along adjacent trophoblastic cell membranes (Fig. 11). They are characterized by the presence of many fine filaments extending on either side into the adjacent cytoplasm. These filaments run parallel to the cell membranes and form a conspicuous border

FIGURES 7 - 68

The plane of section is the same for all figures, that is, cross sections through blastocysts showing the individual trophoblastic cells in longitudinal section. This is indicated in Figs. 7 and 8.

FIGURE 7

Sketch through a cross section through a typical day 12 blastocyst. Microvilli are shown diagrammatically in the region enclosed in the rectangle which also indicates the orientation of Fig. 8. E = endodermal lining of trophoblast; ICM = inner cell mass; Tr = trophoblast.

FIGURE 8

Region of typical day 12 trophoblast. The slightly flattened trophoblastic cells bear microvilli (mv) on that surface facing the uterus and are lined by a layer of endodermal cells (e) with long cytoplasmic processes. Paragon stain; brightfield optics.

FIGURE 9

Section through day 14 trophoblastic cell showing cuboidal shape and cytoplasm rich in ribosomes and granular endoplasmic reticulum. mv = microvillous coating on uterine side of trophoblast; p = long, slender process of endodermal cell (not shown).

FIGURE 10

Region of cytoplasm taken from a day 14 trophoblastic cell. Abundant polysomes (p) and granular ER (RER) are interspersed with occasional lipid droplets (lip). At this stage, the central portions of the lipid droplets often display no osmophilia. The arrows indicate distended ER.

FIGURE 11

Cross section through day 16 trophoblastic tissue showing a typical junction between 2 cells. Desmosomes (d) are located along the length of the adjacent cell membranes and the associated filaments (f) form a conspicuous border on either side. The arrows indicate some filaments which extend for a considerable distance into the cytoplasm of one of the cells. fo = possible fold in plasma membrane; rer = rough (granular) endoplasmic reticulum.

FIGURE 12

Enlargement of indicated region in Fig. 11 showing fine structure of a single desmosome (d). The arrow indicates fine filaments which traverse the intercellular space separating the 2 halves of the desmosome.

FIGURE 13

A desmosome (d) at a junction between the slender cytoplasmic processes of 2 endodermal cells.

roughly 0.25 μ m on either side. In some cases, they also extend considerably farther into the cytoplasm (Fig. 11, arrowheads). Finer filaments traverse the intercellular space separating the 2 halves of each desmosome (Fig. 12, arrow). In addition, the areas of contact between the long cytoplasmic processes of the endodermal cells are strengthened by desmosomes (Fig. 13).

Lipid droplets accumulate in the trophoblastic cytoplasm between days 12 and 16. At day 12, only the occasional cell showed one or possibly two areas of sudan positivity, too weak to photograph, and rarely were structures resembling lipid droplets observed in electron micrographs. Fig. 14 shows a typical day 12 trophoblastic cell; the cytoplasm is devoid of lipoidal inclusions. By day 14, several inclusions structurally suggestive of lipid droplets could be identified in sections of each cell (Fig. 15) and by day 16 their numbers had increased substantially (Fig. 16). The increase in sudan positivity observed in the trophoblast over this interval (Figs. 17 to 20) suggests that these structures are lipid-containing.

The appearance of the lipid droplets, however, differs between days 14 and 16, as observed both ultrastructurally (Figs. 15 and 16) and in the sudan-stained tissue sections (Figs. 17 to 20). At the earlier stage, their boundaries are smooth and the osmiophilic material forms a shell roughly one-third the total radius of each droplet surrounding an electron-lucent core. By day 16, they are somewhat more osmiophilic and usually contain lipid throughout. However, their boundaries are often no longer smooth and appear, instead,

FIGURE 14

Section through a typical day 12 trophoblastic cell, showing the absence of cytoplasmic lipid droplets. ly = lysosome; mv = microvilli; n = nucleus.

FIGURE 15

Portion of a day 14 trophoblastic cell; several lipid droplets are present in the cytoplasm. The label (lip) is centrally placed in the droplets. These electron-translucent regions are devoid of the osmiophilic material which forms a rim around them. ly = lysosome.

FIGURE 16

Day 16 trophoblastic cytoplasm showing the presence of numerous lipid droplets (lip) which differ from those observed at day 14 in 2 ways. They contain osmiophilic material throughout and their boundaries are not smooth. mv = microvilli.

FIGURES 17 - 20

Sud = regions of sudan positivity (lipid).

FIGURE 17

Day 14 trophoblast stained with sudan black B. Phase contrast.

FIGURE 18

Day 16 trophoblast stained with sudan black B. The number of sudan-positive regions in each cell has increased over that observed at day 14. Phase contrast.

FIGURE 19

Another region of day 16 trophoblast treated in the same manner and giving the same information as that seen in Fig. 18. Phase contrast.

FIGURE 20

Portion of trophoblastic-caruncular junction, again stained with sudan black B. ME = maternal epithelium of caruncle; Tr = fetal trophoblast. Phase contrast.

to be retracted in places.

Lysosomal-like organelles of varying appearances populate the trophoblastic cells during this period (Figs. 21 to 23). The presence of acid phosphatase-positive areas of similar size variability in the cytoplasm of GMA-embedded trophoblastic cells observed with the light microscope (Figs. 24 to 26) suggests that these organelles are involved in digestion and this was specifically confirmed when the acid phosphatase-positive regions in day 14 cells were relocated and photographed using the electron microscope (Fig. 27). At day 12, the cytoplasm of virtually every trophoblastic cell contains regions of acid phosphatase activity (Fig. 26) whereas at day 14, the trend is toward larger, more localized areas in most, but not all, of the cells (Fig. 25).

The lysosomes observed in the electron micrographs display a comparable range of sizes. At day 12, they are quite small and several are present in a single cell (Fig. 21) while, over the next 4 days, they tend to increase in size and at day 16 many occupy up to half the cell volume (Fig. 23). While these structures often contain cellular debris such as membrane fragments and chromatin-like material, their other constituents vary with the stage of gestation. Day 12 lysosomes are characterized by an abundance of small, electron-dense bodies about $0.1\text{ }\mu\text{m}$ in diameter (Figs. 21, 28, 29 and 32) while those of days 14 through 16 contain fewer of these bodies and increasing numbers of crystals (Figs. 22 and 23).

FIGURE 21

Portion of day 12 trophoblastic cell showing several typical lysosomes (ly), all of which contain some $0.1\text{ }\mu\text{m}$ bodies (sb).
mf = membrane fragment.

FIGURE 22

Section through day 14 trophoblast showing a lysosome (ly) in which a single crystal (c), of the size and shape always encountered at this stage, is seen. Membrane fragments and other cellular debris are present in this lysosome, but no $0.1\text{ }\mu\text{m}$ bodies.

FIGURE 23

Day 16 trophoblastic cell containing 2 lysosomes (ly), one of which is quite large. Both contain crystals (c) and the larger lysosome also contains chromatin-like material (clm) arranged in a manner reminiscent of the structure of a nucleus.
cm = cell membrane (to give an idea of the size of the cell in relation to the size of the lysosome); n = nucleus.

FIGURE 24

Acid phosphatase reaction (red) on day 14 trophoblastic tissue. n = nucleus. Fast green FCF counterstain; brightfield optics.

FIGURE 25

Day 14 trophoblastic tissue incubated to show localization of acid phosphatase activity. AP = positive reaction for acid phosphatase. Phase contrast optics.

FIGURE 26

Day 12 trophoblastic tissue incubated to show localization of acid phosphatase activity. Acid phosphatase-positive regions are indicated by darker staining in the cytoplasm of all of the trophoblastic cells; the endodermal cytoplasm (e) alone is negative. n = nucleus. Phase contrast optics.

FIGURE 27

Electron micrograph of a GMA-embedded section of day 14 trophoblast previously incubated for acid phosphatase for optical microscopy. The enclosed region is acid phosphatase-positive; the crystal (c) identifies it as a lysosome. n = nucleus.

FIGURE 28

Section through a day 12 trophoblastic cell showing several lysosomes (ly) which contain numerous $0.1\mu\text{m}$ bodies. Several $0.1\mu\text{m}$ bodies (sb) can also be seen in the microvillar region (mv) of this cell. sb = $0.1\mu\text{m}$ small body.

FIGURE 29

Section through day 12 trophoblast showing a single lysosome (ly) containing cellular debris and many $0.1\mu\text{m}$ bodies. Again, several $0.1\mu\text{m}$ bodies (sb) populate the microvillar region (mv). The arrow indicates 3 of these bodies which are possibly being phagocytised.

FIGURE 30

Small bodies (sb) in the microvillar region (mv) of a day 12 trophoblastic cell. The arrow indicates one which has possibly just been phagocytised.

FIGURE 31

Two $0.1\mu\text{m}$ bodies (sb) in the microvillar region of a day 12 trophoblastic cell shown at high magnification so that their substructure, an electron-dense core surrounded by a less electron-dense matrix, can be discerned. It cannot be determined, at this magnification, whether they are surrounded by a single membrane or not.

FIGURE 32

Section through a lysosome (ly) in day 12 trophoblastic tissue which contains $0.1\mu\text{m}$ bodies (sb).

The 0.1 μ m bodies can also be seen in the microvillar regions of the trophoblastic cells, especially at day 12 (Figs. 28 to 31). Occasionally, it appears that some are possibly being phagocytised by these cells (Figs. 29 and 30, arrows). Fig. 31 shows them to be composed of an electron-dense core surrounded by a less electron-dense matrix. Although they do seem, at intervals, to be bounded by an electron-dense line, it cannot be determined at this magnification if this is indeed a membrane. At day 14 they have only been seen among the microvilli in two instances and by day 16 none are observed outside the cells. However, at these later stages, they are still present in some of the lysosomes (Figs. 33 to 36).

Crystals are first observed in the fetal tissue at day 14, at which time they are short (less than 1 μ m) and occur singly within lysosomes (Fig. 37). By day 16 many are considerably larger and they are present in clusters, usually within clearly recognizable lysosomes (Fig. 38), but occasionally just closely bounded by a single membrane (Fig. 42, arrows). In cross section (Figs. 39 and 40), they appear to consist of bundles of 2 or 3 fibrils interwoven at right angles with very regular spacing. This pattern is also indicated, although not as clearly, in the portion of a crystal cut in grazing cross section shown in Fig. 41. This electron micrograph demonstrates another feature which was occasionally observed - that is, crystals in a given section of fetal trophoblast do not always stain with the same intensity.

Similar crystals are also present in the maternal uterine epithelium at days 16, 18 and 22 (Figs. 43 to 46); however they

FIGURE 33

Section through a day 14 trophoblastic cell showing a lysosome (ly) in which are located three 0.1 μ m bodies (sb) and a crystal (c) along with considerable flocculent material.

FIGURE 34

Section through a small lysosome (ly) in a day 16 trophoblastic cell in which a few 0.1 μ m bodies (sb) can be seen.

FIGURE 35

Section through day 16 trophoblast showing a lysosome (ly) containing several recognizable 0.1 μ m bodies (sb).

FIGURE 36

Several small lysosomes (ly) in a day 16 trophoblastic cell, two of which contain recognizable 0.1 μ m bodies (sb).

31

FIGURE 37

Section through a day 14 lysosome showing a single crystal (c) along with considerable cellular debris.

FIGURE 38

Lysosome in a section of day 16 trophoblast which contains many crystals (c) of various sizes and a small amount of cellular debris in the lower left corner.

FIGURE 39

Cluster of crystals (c) within a day 16 trophoblastic cell. The crystals are probably within a lysosome, as some cellular debris is also present (lower left corner of picture), but its limiting membrane cannot be clearly discerned. One crystal is shown in gross section.

FIGURE 40

Cross section of the crystal in Fig. 39 is shown here at a higher magnification (C-c.s.). The interwoven appearance, with small bundles of fibrils oriented at right angles to each other, is evident.

FIGURE 41

Portion of a lysosome in day 16 trophoblastic tissue containing several crystals of which one appears to be in grazing cross section (C-c.s. ?). The varying staining intensities of the crystals suggest that some contain relatively more material than others in this section.

FIGURE 42

A group of crystals in a section of day 16 trophoblast around which a recognizable lysosomal membrane could not be discerned. However, a membrane (arrows) could be seen closely associated with 2 sides of the labelled crystal, (fc).

FIGURE 43

Small cluster of crystals (mc) in a maternal caruncular epithelial cell at day 16 of gestation. Here, the entire cluster is bounded by a single membrane which also encloses some very electron-dense material which may be cellular debris. The possibility that this structure is a lysosome cannot be ruled out.

FIGURE 44

A single crystal (mc) in a section of day 18 (of gestation) maternal caruncular epithelium which is closely bounded by a single membrane (arrows). The crystal is located at the apical end of this cell, close to the microvilli (mv) at the maternal-fetal junction.

FIGURE 45

Three crystals (mc) in the middle to basal region of a day 16 (of gestation) maternal caruncular epithelial cell. They are all closely bounded by single membranes (arrows); however, in the case of the pair located close together, no membrane can be seen along the 2 sides facing each other.

FIGURE 46

A small, membrane-bounded bundle of crystals (mc) in a maternal caruncular epithelial cell at day 22 of gestation. The microvilli at the feto-maternal junction can be seen in the upper right corner.

usually occur singly or in very small groups. While they do not usually appear to be contained within lysosomes (a possible exception is shown in Fig. 43), they are still apparently single membrane-bounded (Figs. 44 to 46).

Although the crystals react positively to all three of the protein stains, naphthol yellow S; mercury-bromphenol blue and aniline blue black (Figs. 49 to 51), they are not removed by the treatment of tissue sections with either papain or protease (Figs. 47 and 48). A comparison of Figs. 47 and 48 indicates that, while the enzyme treatment does remove some of the proteinaceous cellular constituents from the glutaraldehyde-fixed tissue sections, it does not alter the crystals in either size or staining intensity with aniline blue black.

The descriptions given here refer to all of the trophoblastic cells at days 12 to 15 and the majority of those observed at day 16. However, at this last stage examined, a new cell type, the binucleate cell, containing structural specializations for the production and release of a secretory product, is beginning to emerge. While some binucleate cells observed at the early stages do contain many of the cytoplasmic inclusions of the uninucleate trophoblastic cells, such as lipid droplets (Fig. 53) and crystals (Fig. 55), they also possess certain distinguishing features, such as a spherical cell shape (day 16 uninucleate cells are cuboidal to columnar), 2 nuclei which are very smooth in outline (uninucleate cell nuclei can be either smooth or irregular in outline), a well-developed Golgi complex and overall increased cytoplasmic electron density (Figs. 52 to 56). The latter is

FIGURE 47

Section of day 30 trophoblastic-caruncular junction digested with papain for 48 h at 37°C and subsequently stained with aniline blue black for protein. Although the intensity of the general cytoplasmic staining is reduced over that obtained with control sections (Fig. 48), that of the crystals (c) is not. Brightfield optics.

FIGURE 48

Control section of day 30 trophoblastic-caruncular junction incubated in buffer lacking papain and then stained with aniline blue black for protein. The crystals (c) stain with an intensity similar to that observed in Fig. 47. Brightfield optics.

FIGURE 49

Section of day 30 trophoblastic-caruncular junction stained with naphthol yellow S. The crystals (c) react positively to this stain for protein. Phase contrast optics.

FIGURE 50

Section of day 28 trophoblastic-caruncular junction stained with mercury-bromphenol blue for protein. The crystals (c) stain positively. Phase contrast optics.

FIGURE 51

Section of day 30 trophoblastic-caruncular junction stained with aniline blue black. The crystals (c) are positive to this histochemical stain for protein. Phase contrast optics.

10 μ m

(47)

10 μ m

(48)

10 μ m

(50)

10 μ m

(51)

10 μ m

FIGURES 52 - 56

The orientation in these Figs. is such that the feto-maternal junction is below the binucleate cells. This is clear in Fig. 53 in which the microvilli of the feto-maternal junction can be seen.

FIGURE 52

One of the few differentiated binucleate cells (bc) observed in day 16 trophoblastic tissue. Extensive proliferation of the Golgi complex (g) can be seen in that half of the cell facing the feto-maternal junction. The rounded shape of the cell, as well as the 2 separate nuclei (n) is evident.

FIGURE 53

Day 16 trophoblastic cell which appears to be in the process of becoming a binucleate cell (dbc?) in that the cytoplasmic density has increased and proliferation of the Golgi complex (g) appears to be under way. It would appear that this cell contains only one nucleus (n), not only because the section has traversed just one, but also because a survey of many binucleate cells has indicated that once the 2 nuclei have formed, they always appear to have very smooth outlines (see Figs. 52, 55 and 56), in contrast to the one present in this cell. Numerous lipid droplets (lip) are also present in this cell which is resting on and contributing to the microvillous border separating the fetal and maternal layers. Desmosomes (d) are distributed sporadically along the boundary of this cell; however, they are not associated with a very abundant array of filaments.

FIGURE 54

Insert of part of the junction between the possible differentiating binucleate cell shown in Fig. 53 (bc) and its neighbouring uninucleate cell (uc) so that a comparison of the cytoplasmic electron densities can be made. The increased electron density of the binucleate cell cytoplasm in general, is due to the presence of fibrillar material (arrows) such as is present here.

FIGURE 55

Day 18 trophoblastic binucleate cell (bc) containing a crystal (c). n = nucleus.

FIGURE 56

Day 22 trophoblastic binucleate cell (bc) containing extensive Golgi complex, basally (g) and possible secretory granules apically (arrows). Such granules may be responsible for the apical PAS-positivity of these cells at day 30 (Fig. 58). n = nucleus.

FIGURE 57

Day 16 trophoblastic-caruncular junction stained with PAS. PAS-positive material can be seen in a possible binucleate cell (large arrow) and at the microvillar border between the fetal and maternal tissues (small arrows). Phase contrast optics.

FIGURE 58

Day 30 trophoblastic-caruncular junction stained with PAS. PAS-positive material (large arrow) is present in the apical half of a clearly recognizable binucleate cell (n = the 2 nuclei of this cell) and is also at the microvillar border between the trophoblast and the maternal caruncular epithelium (small arrows). Considerably more of this material is present along the microvilli than was observed at day 16 (Fig. 57). Phase contrast optics.

apparently due to the presence of fibrillar material (Fig. 54, arrows). The presence of 2 of these features, an extensive Golgi complex and increased cytoplasmic electron density, in the cell in Fig. 53 suggests that it is in the process of becoming a binucleate cell. However, it would appear that it has not yet attained that state. The single nucleus traversed in this section is very tortuous in outline, in marked contrast to the smooth contours of the nuclei found in mature binucleate cells (Figs. 52, 55 and 56). Hence, while certain of the cytoplasmic developments are already in progress, the division and final differentiation of the nucleus has not yet taken place. Mature binucleate cells also lack the desmosomes which are so prominent along the uninucleate cell junctions (Figs. 52, 55 and 56). Desmosomes can be discerned at points along the boundary of the differentiating cell shown in Fig. 53; however, they are not associated with the conspicuous array of filaments usually encountered in ovine trophoblastic tissue.

At day 16, roughly 1 trophoblastic cell in 20 has developed cytoplasmic specializations such as increased electron density and extensive proliferation of the Golgi complex, indicating that differentiation into a binucleate cell is in progress. However, mature binucleate cells, containing 2 distinct, spherical nuclei in addition to the above-mentioned cytoplasmic developments are considerably more rare - perhaps 1 cell in 100. By days 18 and 22, the proportion of mature binucleate cells has increased to 1 cell in 15 or 20.

PAS staining of the trophoblast-uterine epithelium junctions

at days 16 and 30 shows strong binding of the dye to 2 regions, the binucleate cells (Figs. 57 and 58, large arrows) and the microvillous border separating the fetal and maternal layers (Figs. 57 and 58, small arrows). In the newly-formed binucleate cell (day 16), PAS positivity appears to surround the central nuclei (Fig. 57). In the mature binucleate cells at day 30 (Fig. 58), the PAS staining has become localized and is at the apical ends of the cells, that is, above the nuclei and toward the embryo rather than the maternal uterine tissue. It is possible that the granules indicated in Fig. 56 are responsible for the PAS-positivity. More will be said about this in the discussion.

In every conceptus examined by optical microscopy, the occasional pale-staining cell was observed interspersed among the other trophoblastic cells (Fig. 59). These cells appear to be in the process of disintegrating, for when they were examined ultrastructurally, the presence of numerous large spaces indicated a loss of many of the cytoplasmic components and, furthermore, those which remained were very disorganized (Figs. 62 and 64).

The plasma membrane on the microvillar surface of these cells is lost early in the process of disintegration (Figs. 62 and 64). This may account for the expanded appearance of some of the dying cells in relation to their healthy neighbours (Fig. 60). Nuclei often appear to be one of the last components to lose their organization (Figs. 59, 61 and 66); however, the presence of a clump of chromatin-like material in the disintegrating trophoblastic cell in Fig. 62 suggests that this may not always be the case.

FIGURE 59

Portion of day 16 trophoblastic tissue showing a pale-staining cell in the process of disintegrating (dc) and a second fairly pale-staining cell (ec) which appears to have been engulfed by another trophoblastic cell. Paragon stain; brightfield optics.

FIGURE 60

Portion of day 12 trophoblastic tissue in which a pale-staining disintegrating cell (dc) is located adjacent to an endodermal cell undergoing replication. Paragon stain; brightfield optics.

FIGURE 61

Cluster of dying cells (dc) located in a portion of day 14 trophoblastic tissue. Many of these pale-staining cells have lost a lot of their cytoplasmic constituents; nuclei, however, can still be recognized. Paragon stain; brightfield optics.

FIGURE 62

Day 12 trophoblastic cell in the process of dying (DC). Many spaces in the cytoplasm are evident and a clump of chromatin-like material (clm) appears to be all that is left of the nucleus. Mitochondria (m) can still be recognized; however the ribosomes are very dispersed and the granular ER has formed small, round vesicles. A plasma membrane cannot be seen surrounding this cell.

FIGURE 63

Junction between a dying day 12 trophoblastic cell (dc) and one that is apparently healthy (lc). Desmosomes (d) with their associated filaments are still present; however, the filaments extending into the dying cell are more dispersed than those in the healthy one.

FIGURE 64

A disintegrating day 16 trophoblastic cell (DC) with a healthy neighbouring cell. Lipid droplets (lip) and mitochondria (m) can still be recognized in the dying cell; but the plasma membrane and ribosomes have been lost. Desmosomes (d) still link this cell to the one adjacent.

FIGURE 65

Near-adjacent section to the one used for Fig. 64. It can be seen that the dying cell is only one in a series of healthy trophoblastic cells. Paragon stain; brightfield optics.

FIGURE 66

Nucleus in a day 16 dying trophoblastic cell. Although almost all of the cytoplasmic constituents have been lost, a recognizable nucleus with associated nuclear membrane and nucleoli, still remains.

In this particular instance, there is no indication whatsoever of a nuclear membrane or typical nuclear substructure, in contrast to the 2 dying cells in Figs. 59 and 66 which appear to have reached an even more advanced stage of overall disintegration. Desmosomes, as well, maintain their structural integrity well into the disintegration process (Fig. 63). In many of the dying cells, distorted mitochondria and occasional lipid droplets (days 14 through 16) are still recognizable, but whatever granular endoplasmic reticulum remains is rounded up into vesicles and the ribosomes and polysomes, so abundant in the healthy trophoblastic cells, have become dispersed. In some instances, so many of the cytoplasmic constituents have been lost that a large space, up to the width of a single cell, marks the place where a living cell had once been (Fig. 67).

Disintegrating cells are sometimes observed in close proximity to others which are undergoing replication (Fig. 60). An optical microscopic study of serial sections taken at each stage of gestation (roughly 320 sections at day 14, 170 at day 16 and 100 each at days 12 and 15) indicates that the usual distribution of disintegrating cells is fairly random. A single, or sometimes a pair of dying cells is surrounded on all sides by healthy trophoblastic tissue (Figs. 67 and 68). Only on rare occasions is a cluster of dying cells, such as those shown in Fig. 61, encountered. At all stages examined, roughly 1 cell in 20 to 30 is at some stage of disintegration and, although, as mentioned before, the distribution of dying cells appears basically random, these cells are not always equally spaced. For example, in a single section

FIGURE 67

Serial sections showing that a dying cell in day 16 trophoblastic tissue is adjacent to a healthy cell (arrows). Fig. 67B indicates that so much of the cellular components have been lost that a large space, which may or may not be accentuated by the fixation procedures employed, has developed and is only kept from being a hole right through the blastocyst by the endodermal lining. Paragon stain; brightfield optics.

FIGURE 68

Serial sections-through day 16 trophoblastic tissue. The numbers refer to specific regions in the trophoblast which were followed through the serial sections to see if the dying cells occur singly or in some sort of pattern of rows or clusters. The dying cells at 1, 2 and 4 are adjacent to healthy cells. At region 3, two dying cells are separated by a single healthy cell.

of tissue, one might encounter a row of about 20 trophoblastic cells interspersed with 3 or 4 dying cells (see, for example, Fig. 68) followed by another row of 20 cells, all of which appear completely healthy.

Discussion

The day 12 to day 16 sheep trophoblast has a number of features in common with those of other mammals at comparable stages of development. The blastomeres of the early ovine conceptus (up to day 4) do not remain completely apposed following division, but become a loose cluster of cells with many intercellular spaces (Calarco and McLaren, 1976). Similar development is reported in a number of species including the mouse (Calarco and Brown, 1969; Nowel and Chapman, 1976), rat (Schlafke and Enders, 1967), pig (Norberg, 1973), baboon (Panigel et al., 1975) and rhesus monkey (Hurst et al., 1978) and is further characterized by the presence of microvilli extending from adjacent cells into these spaces and occasionally interdigitating. Calarco and Brown (1969) suggested that this interdigitation holds the blastomeres more firmly together during both the morula and blastocyst stages and possibly this role could also be applied to the interdigitations observed throughout the period of rapid blastocyst expansion in the sheep, even though these cells are more firmly interconnected by other cell to cell interactions.

The observation of well-developed spot desmosomes linking adjacent trophoblastic cells throughout the interval studied is in agreement with the work of Wintenberger-Torrès and Fléchon (1974) and, in fact, these junctional specializations first begin to appear in the blastocyst at the time of its initial formation at about day 6.5 (Calarco and McLaren, 1976). Spot desmosomes have

been observed in the trophoblastic layer of many other mammalian blastocysts, such as those of the mouse (Nowel and Chapman, 1976), rat (Schlafke and Enders, 1967), armadillo and Mexican funnel-eared bat (Enders, 1971), pig (Hall et al, 1965; Norberg, 1973), baboon (Panigel et al, 1975) and rhesus monkey (Hurst et al, 1978).

The spot desmosomes linking these trophoblastic cells are very similar to those found in intestinal epithelium (Hull and Staehelin, 1979). In both cases, the tonofilaments extending from each desmosome run in bundles parallel to the 2 plasma membranes and, in some instances, extend well into the adjacent cytoplasm. It has been proposed that these filament bundles provide a strong structural framework for the cell by interconnecting desmosomes on all sides (Staehelin and Hull, 1978; Hull and Staehelin, 1979). Finer filaments traverse the intercellular spaces within desmosomes from both intestinal epithelium and ovine trophoblast and these have been implicated in the provision of firm cell to cell adhesion (Staehelin and Hull, 1978; Hull and Staehelin, 1979).

It is possible that other types of intercellular junctions, specifically tight and gap junctions, are also present along the adjacent trophoblastic cell boundaries. Unfortunately, the electron micrographs are not of sufficiently high resolution for this to be elucidated. In longitudinal section, tight junctions consist of regions of contact between adjacent cell membranes, fortified by sealing strands, alternating with areas in which the two membranes are separated by a small space (Staehelin and Hull, 1978). Their primary role is to allow a group of cells to maintain an inner environment chemically different from the

external one (Staehelin and Hull, 1978); in the case of a blastocyst, such structures could aid in the accumulation and maintenance of a blastocoel fluid of different composition from the uterine fluids, bathing the conceptus.

Gap junctions, on the other hand, consist of a region along two adjacent cell boundaries in which the intercellular space has been reduced from about 250 to 30 angstroms (Staehelin and Hull, 1978). Since, in most tissue sections, such a small space would not even be seen and the two membranes would appear to be in direct contact, gap junctions are often mistaken for tight junctions (Staehelin and Hull, 1978). In these areas of close membrane apposition, gap junction particles make up tiny intercellular channels which allow the rapid flow of small molecules, such as sugars, amino acids, steroid hormones and cyclic AMP, between cells. In the developing embryo, this would permit the regulation of the growth and differentiation of a large number of cells so that all developmental stages were encountered in an organized fashion (Staehelin and Hull, 1978).

Regions of close apposition or fusion have been observed in electron micrographs of the plasma membranes of days 12 to 16 trophoblastic cells. The presence of tight junctions in the 16-cell ovine conceptus has been postulated (Calarco and McLaren, 1976). Gap junctional communication has been reported in the mouse conceptus between the 8-cell and blastocyst stages by the simultaneous demonstration of ionic coupling and cell to cell transfer of the low molecular weight dye, sodium fluorescein (Lo and Gilula, 1979). At the magnifications used for this study,

it was impossible to tell whether the regions of junctional specialization contain the button-like points of contact between membranes characteristic of tight junctions or are areas of uniformly close apposition connected by cross-bridges, that is, gap junctions. It is even possible that both types of junctions are present, since each could serve a useful role in blastocyst development. The elegant physiological experiments of Lo and Gilula (1979) using cultured blastocysts would be very useful in distinguishing between these two types of junctions.

The endodermal cells observed throughout this period originate from the inner cell mass beginning at about day 8 (Wintenberger-Torrès and Fléchon, 1974) and migrate from this source to form a complete inner lining within the next 2 to 3 days (Green and Winters, 1945; Boyd and Hamilton, 1952; Bryden *et al.*, 1972; Wintenberger-Torrès and Fléchon, 1974). The differentiation of a complete endodermal layer has also been reported in the pre-implantation blastocysts of the cow (Chang, 1952) and the pig (Hall *et al.*, 1965), two species whose embryos reach an advanced stage of development before establishing placental contact. Although the presence of desmosomes linking endodermal cells has not been reported in these species, in all probability, an investigation of the ultrastructure of these cells would indicate that such specialized junctions have been developed, as in the sheep, to help maintain cellular structure and adhesion.

The presence of polyribosomal clusters is a characteristic of developing mammalian blastocysts in general and can be attributed to a requirement by these rapidly growing blastocysts

for a continuous supply of structural proteins.

Two possible uterine sites of lipid release for uptake by the day 12 to 16 blastocyst are the intercaruncular glands and the uterine epithelium. The former produce a secretion, uterine milk, in which the presence of fat globules, among other nutrients, has been demonstrated (Bonnet, 1882; Jenkinson, 1906). In addition, lipid droplets populate the day 16 caruncular epithelial cells (Carnegie, unpublished observation) and it is possible that these, too, eventually end up in the trophoblastic cells. The histochemical identification of lipid in ovine trophoblastic cells beginning at day 12 is in agreement with the ultrastructural observations of Wintenberger-Torrès and Fléchon (1974). The presence of lipid droplets, based on the satisfaction of ultrastructural criteria, has been reported in a number of mammalian blastocysts including the rat (Schlafke and Enders, 1963; Schlafke and Enders, 1967), rabbit (Hesseldahl, 1971), golden hamster (Parkening, 1976), rhesus monkey (Hurst *et al.*, 1978) and pig (Hall *et al.*, 1965) and further substantiated by the demonstration of sudanophilia in the armadillo (Enders, 1962), mouse (Smith and Wilson, 1971) and cow (Greenstein *et al.*, 1958). Blastocysts of species such as the pig, cow and sheep, which elongate tremendously before establishing placental contact with the mother, require a large nutrient source during this time of rapid growth. This may be at least in part supplied by lipids released into the uterine lumen.

The interpretation of the different appearances of the lipid droplets at days 14 and 16 should be made cautiously, in that

lipid droplet morphology is dependent upon such facets of the fixation procedure as failure of the osmium tetroxide to penetrate adequately (Fawcett, 1967) and exposure of the tissue to aldehyde fixatives prior to immersion in osmium tetroxide (Bloom and Fawcett, 1968). Osmium tetroxide fixes lipoidal material by reacting with the double bonds of unsaturated lipids to form an additive compound (Baker, 1958). For this reason, droplets containing highly unsaturated lipids are more osmiophilic and hence show better preservation and greater darkening than those composed of more saturated fatty material. This would suggest that the lipid droplets found in day 16 trophoblastic tissue, which appear more osmiophilic than those at day 14, contain a higher proportion of unsaturated lipid.

Osmium tetroxide is a fixative which penetrates tissue slowly (Hopwood, 1970) and, unless very small (1 mm^3) pieces are used, this can also be a factor. Lipid droplets which have not encountered enough osmium tetroxide may have their unfixed centres extracted during the subsequent dehydration and embedment procedures and, in section, appear as rings of osmiophilic material surrounding clear centres (Fawcett, 1967). This was repeatedly encountered in day 14 trophoblastic tissue and may again point to a higher degree of saturation of the lipid for the following reason. At both days 14 and 16, the trophoblast consists, for the most part, of a single layer of cells and so the degree of penetration of the osmium tetroxide into the tissue fragments should not vary between these two stages, at least on the basis of depth of tissue to be traversed. However, droplets

composed of primarily saturated lipid bind less osmium tetroxide and may even be less permeable to penetration by the fixative and they would finally appear, in thin section, as electron-lucent cores surrounded by only moderately osmiophilic material. The retracted appearance of the lipid droplets in day 16 trophoblastic tissue cannot be explained at this time but appears suggestive of shrinkage of the droplets during subsequent processing.

Digestive activity is attributed to several mammalian blastocysts on the basis of the presence of lysosomes in their cells (in the mouse, El-Shershaby and Hinchliffe, 1974; golden hamster, Parkening, 1976; rabbit, Abraham et al, 1970 and sheep, Wintenberger-Torrès and Fléchon, 1974), multivesicular bodies (in the rat, Schlafke and Enders, 1963; Schlafke and Enders, 1967; Wu and Meyer, 1974) or the histochemical demonstration at the light microscope level of cytoplasmic acid phosphatase activity (in the mouse, Christie, 1967; Smith and Wilson, 1971; golden hamster, Parkening, 1976 and rabbit, Abraham et al, 1970). It has been suggested that in 3 of these species, the golden hamster, rabbit (Christie, 1967) and sheep, these lysosomes are involved in the digestion of food material to supply the energy requirements of the growing blastocyst. In the case of the sheep, such material is certainly available during early gestation. Glandular secretions are released into the ovine uterine lumen which contain, in addition to the lipid droplets already mentioned, extravasated red blood cells, connective tissue elements, bits of uterine epithelium and crystals (Amoroso, 1952). In the mouse, embryonic

lysosomes are associated with the digestion of cellular debris resulting from the death of primarily the inner cell mass, but also some of the trophoblastic cells (Smith and Wilson, 1971; El-Shershaby and Hinchliffe, 1974).

Two distinctive lysosomal constituents, the $0.1\mu\text{m}$ bodies and the crystals, may well be of maternal origin, the former because they are initially observed in great abundance in the microvillar region as well as in the lysosomes and the latter because they are also present in the uterine epithelium of both the nonpregnant ewe (Guillomot, 1979) and at day 16 of gestation. Wintenberger-Torrès and Fléchon (1974) reported the presence of $0.1\mu\text{m}$ round corpuscles in the microvillar border of the ovine conceptus at day 12 only. It appears that they are also accumulated in lysosomes at this time and then gradually broken down or converted into other structural forms over the ensuing 4 days. These small bodies are very similar in size and structure to dense-cored vesicles which have been reported in the primordial germ cells of the mouse (Jeon and Kennedy, 1973) and the rat (Eddy and Clark, 1975) and in the developing pineal gland of the hamster (Sheridan and Walker, 1975). In the latter organ, they are postulated to contain secretory product (Sheridan and Walker, 1975).

Wintenberger-Torrès and Fléchon (1974) also reported the phagocytosis of cilia by day 10 trophoblastic tissue. This was not observed between days 12 and 16 of gestation.

The cytoplasmic crystals or "Stabchen" were first described in the ovine trophoblast by Bonnet (1888) and have since been mentioned in a number of studies of the ultrastructure of the sheep

trophoblast or developing placenta (Amoroso, 1952; Davies and Wimsatt, 1966; Wintenberger-Torrès and Fléchon, 1974; Myagkaya and Vreeling-Sindelarova, 1976). Their presence is not confined to early gestation for they have also been observed in lysosomes as late as day 145 (Myagkaya and Vreeling-Sindelarova, 1976). Nor are they a feature of embryonic tissue alone for this study has shown that they are also present in the maternal epithelium at day 16 of gestation and they have been observed in the nonpregnant ovine uterine lining (Guillomot, 1979).

Crystals do not appear to be characteristic of trophoblastic tissue in general. They have been reported in the blastocysts of only 3 other species, the mouse (Enders, 1971; Calarco and Szollosi, 1973), baboon (Panigel et al, 1975) and rabbit (Hadek and Swift, 1960; Enders, 1971; Daniel and Kennedy, 1978). They have been most extensively studied in the rabbit where they have also been observed in the progesterone-stimulated uterine endometrium (Nakao et al, 1971; Hoffman et al, 1975) and it has been determined that their production by the blastocyst requires some component of the uterine environment (Van Blerkom et al, 1973; Daniel and Kennedy, 1978). When viewed in cross section, the rabbit, mouse and ovine crystals have different appearances. Rabbit trophoblastic crystals appear to consist of numerous discrete subunits which are rounded in cross section and are revealed by longitudinal section to be long, slender cylinders (Daniel and Kennedy, 1978). Those of the mouse, while constructed of some sort of interwoven meshwork similar to that of the sheep, are considerably more loosely organized and extremely variable in

size and outline, as apposed to compactly needle-shaped (Enders, 1971). Like those of the sheep, rabbit crystals are membrane-bound; however, neither rabbit nor mouse crystals are contained within large lysosomes but, instead, are often in close proximity to cytoplasmic organelles such as ribosomes and endoplasmic reticulum (Enders, 1971; Daniel and Kennedy, 1978). Insufficient information is available on the baboon crystals for structural comparisons with the other species.

The resistance of the crystals to digestion by proteolytic enzymes is not necessarily indicative of an absence of protein in their structure. The incubations were performed on tissue sections whose proteins had been cross-linked by fixation in glutaraldehyde. Hence, it is quite possible that any proteins present in the crystals, by either being cross-linked to each other or to possible surrounding non-proteinaceous material, would not be fragmented and subsequently removed by the enzyme treatments.

The significance of the crystals in any of these species has yet to be determined; however speculations have been made ranging from viral crystalline arrays in the mouse (Calarco and Szollosi, 1973) to membrane-bounded bundles of microtubules in the rabbit (Daniel and Kennedy, 1978) to breakdown products of erythrophagocytosis in the sheep (Myagkaya and Vreeling-Sindelarova, 1976). Since the crystals do differ in ultrastructure between these 3 species, there is no reason why they should have the same function. The erythrophagocytosis theory proposed by Myagkaya and Vreeling-Sindelarova (1976), which is based on the study of

mature ovine placentomes toward the end of gestation, is attractive in that the developing blastocyst must have a source of iron in order to produce its own red blood cells and, eventually, a circulatory system. The presence of extravasated maternal red blood cells has been reported in ovine uterine milk (Amoroso, 1952) and some of the larger postulated "lipid droplets" in trophoblastic tissue may well be red blood cells. Ultrastructurally, it is difficult to distinguish between the two. Unfortunately, energy dispersive X-ray analysis performed on tissue sections at Dr. J. Lott's laboratory, McMaster University, indicated that no iron was retained in the sections following fixation and embedment for electron microscopy and so the theory that the crystals might contain a breakdown product of maternal haemoglobin or that the trophoblast is ingesting maternal red blood cells between days 12 and 16 of gestation could not be pursued at this time.

The presence of crystals in the ovine uterine epithelium and in the uterine milk suggests that their appearance in the trophoblast results from their uptake by that tissue from these two sources. However, their large size and the finding of longer crystals at day 16 than at day 14 favours their synthesis by the trophoblast over accumulation by phagocytosis. The same theory has been proposed for the rabbit (Daniel and Kennedy, 1978), the other species in which the presence of crystals in both embryonic and maternal tissues has been demonstrated.

Binucleate cells, or giant cells as they are sometimes called, have been described in the fetal placental tissue of 3 ruminants,

the sheep (Wimsatt, 1951; Davies and Wimsatt, 1966; Lawn et al, 1969), goat (Lawn et al, 1969) and cow (Wimsatt, 1951; Greenstein et al, 1958; Bjorkman, 1969; King et al, 1979). The observations made in this study are in agreement with those of Davies and Wimsatt (1966) that ovine binucleate cells contain lipid droplets and crystals early in the process of differentiation, but not once they are mature. The absence of desmosomes along the boundaries of mature binucleate cells has also been reported (Boshier and Holloway, 1977). That ovine binucleate cells first appear at day 16 or 17 of gestation has also been reported by Wimsatt (1951) and by Boshier (1969) and day 17 has also been found to be the time of first appearance of bovine binucleate cells (Wimsatt, 1951; Greenstein et al, 1958).

The rounded cell shape, hypertrophied Golgi complex and increased cytoplasmic density are characteristic of ruminant trophoblastic binucleate cells (Bjorkman and Bloom, 1957; Davies and Wimsatt, 1966; Lawn et al, 1969; Boshier and Holloway, 1977), as is PAS positivity (Greenstein et al, 1958; Boshier, 1969). The polarity of these cells, with the elaborate Golgi complex and most of the mitochondria toward the feto-maternal junction and membrane-enclosed granules on the opposite side of the nuclei has been previously reported in mature ovine placentomes (Davies and Wimsatt, 1966; Lawn et al, 1969; Boshier and Holloway, 1977).

The localization of the PAS-positive staining is also polarized; it is on that side of the cell away from the feto-maternal junction. At first this might seem confusing in that the PAS-positivity is located on the opposite side of the cell to the Golgi complex.

However, studies have indicated that granules roughly $0.5\mu\text{m}$ in diameter are produced in great numbers by the binucleate cells and that they accumulate in the apical ends of these cells (Wimsatt, 1951; Davies and Wimsatt, 1966; Lawn *et al*, 1969; Boshier and Holloway, 1977). Granule production appears to have just begun in the binucleate cell from day 22 trophoblast shown in Fig. 56. The suggestion has been made that they are storage granules which contain glycoprotein (Boshier and Holloway, 1977), based on the work of Boshier (1969) which has shown them to be resistant to diastase digestion. This would account for the apical PAS-positivity.

In the mouse, embryonic lysosomes are associated with the digestion of cellular debris resulting from the death of primarily inner cell mass, but also of some trophoblastic cells (Smith and Wilson, 1971; El-Shershably and Hinchliffe, 1974). Cell death during early blastocyst development is not widely reported in the literature. El-Shershably and Hinchliffe (1974) estimated that roughly 10% of the blastocyst cells of the mouse die and are phagocytised by their neighbours. This occurs between 86 and 105 h of gestation, prior to loss of the zona pellucida, so that any confusion with maternal cells can be ruled out. This is the time of the initial activation of the embryonic genome and the differentiation, functionally, of the 2 cell populations, inner cell mass and trophoblast. For this reason, El-Shershably and Hinchliffe (1974) postulated that selected embryonic cells died as a result of genetic inadequacies which made them incapable of responding to a stimulus to differentiate.

Trophoblast cells which differ in electron density have been reported in 2 species, the baboon (Panigel et al, 1975) and the sheep (Wintenberger-Torrès and Fléchon, 1974). Wintenberger-Torrès and Fléchon (1974) divided days 8 and 10 sheep trophoblastic cells into 2 groups, "clear" or "dark". Little description of the former was given; however a comparison of micrographs indicates the presence of some similarities between their "clear" cells and the disintegrating cells described in this paper, such as a loss of much of the cytoplasmic ground substance. Their "clear" cells sometimes lacked microvilli; in this study, it was consistently found that microvilli were lost early in the process of disintegration. Panigel et al (1975) observed that baboon trophoblastic cells, as well, differ in the densities of the microvillous borders. However, they made no comparisons between this observation and a previous one that some trophoblastic cells appear more electron-dense and differentiated than others.

The death of a small proportion of trophoblastic cells is also reported in the cow (Bjorkman, 1969), but at a later stage of gestation and is associated with remodelling of the placentome.

Hence, trophoblastic cell death in other species is attributed to one of two factors, either a genetic defect which renders the cell incapable of differentiation or tissue remodelling requiring the death of certain cells so that a particular overall shape can be attained. Although cell death occurs in the sheep blastocyst at a later stage (6 to 10 days after the differentiation of the inner cell mass and trophoblast) than in the mouse (El-Shershaby and Hinchliffe, 1974), it is nevertheless still a time

of cellular differentiation. Two developments occurring at this time are the emergence of a new trophoblastic cell type, the binucleate cell (Wimbatt, 1951; Boshier, 1969) and the change in shape of the uninucleate trophoblastic cells from cuboidal to columnar. Possibly some of the cells are incapable of making these changes and can no longer survive.

There is also some reshaping of the blastocyst occurring at this time. Scanning electron microscopy has shown that villi extend from the day 15 blastocyst into the intercaruncular uterine glands, possibly to help anchor the conceptus as well as to ensure maximal uptake of uterine secretion (Guillomot *et al*, 1980). Regions of day 16 trophoblast consisting of more than one layer of cells have been observed (Carnegie, unpublished observations). Selective cell death could play a role in the shaping of these processes.

Random location of dying cells would support a genetic defect theory whereas cell death for remodelling purposes requires cell death that is more localized. It may be that in the sheep both of these are in effect simultaneously, since examples of both random distribution (much more common) and clustering of disintegrating cells are found.

But is cell death a normal feature of early ovine development or was it observed in these blastocysts because they were unhealthy and in the process of being resorbed? Two facts argue against this being a series of observations on abnormal embryos. First, isolated disintegrating cells were found scattered throughout healthy trophoblastic tissue in every conceptus examined. Secondly,

dead cells were sometimes located in close proximity to dividing cells, a situation one would not expect to find in an embryo undergoing resorption. One experiment could be performed which would settle the question. Rowson and Moor (1966) have transferred day 13 ovine blastocysts which have sustained a loss of a small amount of trophoblastic tissue to recipient ewes who have successfully carried them to term. It should therefore be possible to remove, fix and embed a small piece of trophoblastic tissue, examine it for the presence of dead cells and, at the same time, return the blastocyst to an acceptable uterine environment and see if development into a normal lamb results. It would also be interesting to look for evidence of cell death in other species, particularly other ruminants, in which the type of placentation is the same and in which the blastocysts undergo tremendous elongation prior to implantation.

Part 2. The Immunofluorescent Localization of Ovine Chorionic Somatomammotropin in the Days 14 to 55 Ovine Trophoblast.

Introduction

Ovine chorionic ~~somatomammotropin~~ (oCS), formerly called ovine placental lactogen (oPL), is the name given to the protein hormone produced by the placenta of the sheep which has both growth-promoting and lactogenic properties. It was the latter ability, that of placental lactogens to stimulate the development of the mammary glands during gestation, which led to their initial discovery in ruminants.

Buttle et al (1972) measured total plasma lactogenic activity in pregnant and lactating goats by a rabbit mammary gland culture assay and compared the levels estimated in this way (a rating of 1 to 4 was assigned based on histological determination of secretory response of the mammary glands) to the plasma concentrations of prolactin (PRL) measured by radioimmunoassay (RIA). During lactation, they found a good correlation between plasma lactogenic activity and plasma concentrations of PRL, the traditional lactogenic hormone. However, between the 9th and 15th weeks of gestation, in 4 out of 5 goats, while the plasma demonstrated lactogenic activity corresponding to PRL concentrations of between 100 and 500 ng/ml, the actual levels measured by RIA were between 0.9 and 26.0 ng/ml. Hence, while prolactin still appeared to be the lactogenic hormone following parturition,

22
OF/DE

the possible existence of a second hormone functioning during gestation to prepare the mammary glands for their role in the nourishment of the young had to be recognized. Buttle et al (1972) pointed out that the peak in "extra" lactogenic activity (about day 63 to day 105) correlates well with the time of rapid growth of the lobulo-alveolar tissue of the mammary glands (days 70 to 100) and their further suggestion that this second lactogenic hormone is of placental origin was based on earlier studies by Forsyth (1972) which had shown that goat placental tissue, in vitro, is capable of stimulating mouse mammary gland explants. By this time placental lactogens had already been reported in humans, baboons and monkeys (Josimovich and MacLaren, 1962; Kaplan and Grumbach, 1964; Friesen, 1965) and rats and mice (Ray et al, 1955; Cerruti and Lyons, 1960; Kohmoto and Bern, 1970). It now appeared that at least one representative of the ruminants could be added to the growing list.

Some of the earlier studies brought to light another characteristic of this placental hormone. Josimovich and MacLaren (1962), using an antibody prepared to purified human growth hormone (hGH) and an Ouchterlony double diffusion agar gel system showed that if hGH and human retroplacental serum (serum from placental blood taken at the time of parturition) were placed in adjacent outer wells and the anti-hGH in the central well, they would get a reaction of partial identity between the 2 antigens. These results were later repeated and confirmed by Kaplan and Grumbach (1964). Josimovich and MacLaren (1962) interpreted this to mean that there existed in the

placental serum a hormone which had some, but not all, antigenic sites common to hGH. A crudely purified preparation of this hormone was at least 50% as effective as NIH sheep prolactin in the pigeon crop assay and pseudopregnant rabbit test (induction of milk production), two traditional bioassays for lactogenic activity (Josimovich and MacLaren, 1962). But, despite its apparent structural similarities to hGH, it showed very little growth-promoting ability by the rat tibia test (Josimovich and MacLaren, 1962; Kaplan and Grumbach, 1964).

The structural similarities between hGH and hPL (human placental lactogen) became even more apparent after the 2 hormones had been amino acid-sequenced. Sherwood et al (1971) found that the hormones are identical in 85% of their corresponding positions. They are roughly equivalent in their lactogenic properties but, as indicated previously, hPL is less than 1% as effective as hGH in promoting growth. The authors concluded that lactogenic activity must be associated with a region of the polypeptide chain identical in the 2 hormones, while growth promotion is in a region where some differences in amino acid sequence exist.

By now, placental lactogens have been reported in a number of other species including the sheep, cow, fallow deer, hamster, guinea pig and chinchilla (Forsyth, 1973; Kelly et al, 1973; Kelly et al, 1974; Talamantès, 1975; Kelly et al, 1976). These hormones are initially identified by demonstrating the presence of a substance in plasma partially purified by gel filtration which is active in both the lactogenic and the growth hormone

radioreceptor assays (RRA's). In the former, the ability of the hormone to displace radioactively-labelled prolactin from mammary gland membrane fragments is monitored; growth promoting activity is measured in terms of the displacement of labelled growth hormone from liver membrane fragments (eg. Kelly et al, 1976).

Four different groups have isolated and purified oCS and reported some of its properties (Handwerger et al, 1974; Handwerger et al, 1975; Martal and Djiane, 1975; Chan et al, 1976; Fellows et al, 1976 (Handwerger group); Hurley et al, 1977a and b (Handwerger group); Martal and Djiane, 1977a and b; Reddy and Watkins, 1978a). RRA's using liver and mammary gland membranes have clearly demonstrated the combined growth promoting and lactogenic properties of the purified hormone preparations (Handwerger et al, 1974; Chan et al, 1976; Reddy and Watkins, 1978a); however differences do exist between some of them as to the ratios of the 2 activities. The hormone purified by Chan et al (1976) has twice as much lactogenic as growth promoting activity (1.3 U/mg), whereas that isolated by Handwerger et al (1974), while roughly equivalent in lactogenic stimulation, was only about one-fifth as effective as the GH-RRA.

• OCS is a small protein of molecular weight 22 - 24,000 (Handwerger et al, 1975; Martal and Djiane, 1975; Chan et al, 1976; Hurley et al, 1977b), about the same size and with the same electrophoretic mobility as oGH (Handwerger et al, 1975; Martal and Djiane, 1975). It has been shown to stimulate mammary gland development and casein synthesis in the rabbit (Handwerger

et al., 1974; Chan et al., 1976) and to promote growth, as indicated by total weight gain or growth of tibial cartilage, in the hypophysectomized rat (Chan et al., 1976; Martal and Djiane, 1977b). As would be expected, initial sequencing studies have indicated that it shares structural features with both ovine growth hormone (oGH) and ovine prolactin (oPRL) (Hurley et al., 1977a and b). However, its role or roles in the ewe during gestation have yet to be determined, especially considering that it has been extracted from fetal membranes as early as day 16 of pregnancy (Martal and Djiane, 1977a; Chan et al., unpublished observation), a stage far too early to require the presence of a lactogenic hormone, but one at which the presence of a luteotrophic hormone is essential. It has also been shown for another ruminant, the cow, that a placental lactogenic hormone is present in conceptuses as early as day 17 of pregnancy (Flint et al., 1980).

By RIA, oCS is first detectable in maternal blood plasma by days 40 to 50; the concentration rises throughout gestation to a peak usually 5 to 10 days before parturition, declines steadily until term (Handwerger et al., 1975; Handwerger et al., 1977; Chan et al., 1978a) and decreases very rapidly following parturition (Handwerger et al., 1977; Chan et al., 1978a). It can be detected in allantoic fluid as early as day 18, peaks in concentration between days 35 and 50 and is present, but in decreasing concentrations throughout the rest of gestation (Chan et al., 1978a). When the levels of oCS in fetal and maternal blood are compared, more is present in the fetal circulation between days

46 and 70, whereas the situation is reversed from days 110 to 132 of pregnancy.

An investigation of oCS binding to various ovine tissues showed that it is taken up by the liver, adipose tissue, ovary, corpus luteum and uterus of the pregnant or nonpregnant ewe and by the fetal liver (Chan et al, 1978b) with maximal binding to maternal liver and fat. Binding to these tissues was inhibited by oGH, but not by oPRL or oLH (luteinizing hormone). One very surprising result was a complete lack of binding of oCS to the mammary gland, despite the lactogenic effects of oCS which have been demonstrated in the rabbit. Chan et al (1978b) were unable to explain this discrepancy but stressed the importance of being aware of species differences (eg. testing an ovine hormone using rabbit tissues) when interpreting the results of hormone binding studies..

The high uptake of oCS by the maternal adipose tissue prompted Chan and coworkers (1978b) to suggest a role in fat metabolism, specifically the stimulation of the release of free fatty acids for use by the maternal tissues so that glucose, the primary energy source of the developing fetus, would be spared. However, experiments carried out by Handwerger et al (1975; 1976), monitoring the effects of oCS injections given to both pregnant and nonpregnant sheep on the plasma levels of free fatty acids, insulin, glucose and amino nitrogen showed a sharp decline in circulating fatty acids within 1 h of hormone administration, apparently indicating a stimulation of fat storage as opposed to fat utilization..

Several interesting results of these experiments demonstrate the need for further research in this area, perhaps looking at long term effects of oCS on maternal metabolism. First, while plasma free fatty acid levels did initially drop following oCS administration, they also subsequently rose and reached baseline levels by 6 h post injection. Secondly, oCS appears to have some effect on insulin release, in that plasma insulin levels dropped rapidly following oCS injection, but then rose very quickly, reaching baseline levels by under 2 h post injection and 150% above baseline levels 8 h after the hormone had been given. Insulin promotes fat storage (Turner and Bagnara, 1971) and may well be playing a role in the decline in circulating free fatty acids observed by Handwerger et al (1975; 1976). Another possibility, if oCS is indeed promoting fat storage (either directly or indirectly, through insulin), is that such a role would ensure the deposition of adequate fat reserves which could then be mobilized during lactation. A third rather surprising observation made by Handwerger et al (1976) was that, over the initial 8 h period following oCS administration, there appeared to be no difference between pregnant and nonpregnant sheep as to the effects of oCS on the plasma levels of the 4 factors they measured.

Two other tissues showing significant uptake of oCS, the ovary and corpus luteum, were also of great interest to Chan et al (1978b). In the ewe, an active corpus luteum secreting progesterone is required for the maintenance of pregnancy up to about day 50 (Denamur and Martinet, 1955). During a normal estrous

cycle, if an egg is not fertilized, the corpus luteum begins to regress at about day 14 and is completely gone, and the next estrus under way by day 17 or 18 (Hansel and McEntee, 1977).

Hence, if fertilization has occurred, a signal from the developing embryo must be received by the mother before day 14 to ensure the extension of the lifespan of the corpus luteum so that normal placental attachment and fetal development can occur. Experiments involving embryo removal at early stages of gestation have indicated that the corpus luteum is sensitive to the presence of a developing blastocyst as early as day 12 post coitum (Rowson and Moor, 1967). The possibility that oCS is this embryonic signal has been raised (Chan et al, 1978b).

OCS can be extracted from fetal membranes as early as day 16 of pregnancy (Martial and Djiane, 1977a; Chan et al, unpublished observation) and could possibly be present earlier, but at concentrations below the sensitivity of the assay method. Certain experimental results, however, argue against such a theory. The infusion of purified oCS (Martial and Djiane, 1977a) or of homogenates of day 25 embryos (Rowson and Moor, 1967) into the uterine lumen does not prolong the life of the corpus luteum whereas the infusion of day 14 or 15 embryo homogenates does (Rowson and Moor, 1967). OCS is clearly present in the day 25 conceptus (Martial and Djiane, 1977a; Chan et al, unpublished observation) and is possibly present as early as day 14 or 15. But even though it does appear to be present at the right time, the results of the infusion experiments suggest that it is not responsible for the rescue of the corpus luteum.

The determination of that stage at which oCS is first present in the developing sheep conceptus may be a logical initial step toward elucidating its role in early gestation. A method employing the binding of a fluorescently-labelled antibody to oCS to tissue sections which could then be visualized using a microscope equipped with epifluorescence optics may provide a more sensitive means than radioimmunoassay of determining just how early in gestation this hormone is found in the outermost embryonic membrane, the trophoblast.

Materials and Methods

The materials and methods used for this part of the research project have been published (Carnegie et al, 1980; see Appendix C). Listed here are the additional methods not presented in the published paper.

1. Collection and Handling of Tissue

At days 12, 14 and 16 of gestation, crossbred ewes were slaughtered and the uteri immediately removed and flushed with phosphate buffered saline, pH 7.4 (PBS) to obtain the products of conception. At day 12, the entire conceptus (roughly 3 mm in diameter) was transferred to ice-cold 3% glutaraldehyde (in 0.1 M Sorenson's buffer, pH 6.8) while at the 2 later stages, pieces of chorionic membrane about 2 mm X 2 mm were cut and placed in either cold 3% glutaraldehyde or 4% paraformaldehyde. Fixation in all cases was for 0.5 h and followed by thorough washing in Sorenson's buffer and dehydration and low temperature embedment in glycol methacrylate (Carnegie et al, 1980; see Appendix C).

At day 55, pieces of tissue were taken at the junction of fetal chorion and caruncular epithelium and fixed and embedded in glycol methacrylate (GMA) as previously described (Carnegie et al, 1980; see Appendix C). In addition, samples of unattached chorion were removed from intercaruncular areas (Figs. 1 and 2) and handled in the same manner as that obtained at the 3 earlier

stages.

At days 14 and 28, pieces of corpus luteum roughly 1 mm³ were cut, fixed 0.5 h in ice-cold 3% glutaraldehyde and embedded as above. Table 3 summarizes those tissues investigated for the presence of oCS.

2. Tissue Sectioning and Incubation

Sections (1 - 2 μ m) were cut and attached to glass slides as previously described (Carnegie et al, 1980; see Appendix C) with one addition. At day 12, 4 and 6 μ m thick sections were also prepared, in an effort to determine if even minute quantities of oCS were present. The incubation procedures employed (rabbit anti-oCS followed by FITC-labelled goat anti-rabbit γ globulin) have already been reported and always included a control slide which omitted exposure to the first antiserum (Carnegie et al, 1980; see Appendix C).

3. Preincubation Control

Two control experiments were conducted using sections of days 28 and 55 chorion in which the first antiserum, rabbit anti-oCS, was incubated with a known concentration of oCS antigen for 18 h at 4°C prior to placement on the tissue sections. The remaining incubation steps were followed unchanged and the antibody binding to the tissue was compared with that of near-adjacent sections which had undergone the usual incubation procedure with the same dilution of antiserum. The ratio of

Table 3. Summary of tissues incubated for oCS.

Animal number	Day of gestation	Tissue studied for oCS localization	oCS positivity
74-1736	12	chorion*	+
74-2223	14	chorion** corpus luteum	+ -
74-2416	14	chorion**	+
69-1161	16	chorion**	+
23-3-77	20	caruncular chorion	+
75-0723	28	corpus luteum	-
76-0228	28	caruncular chorion	+
76-0227	55	caruncular chorion intercaruncular chorion	+ +
76-0409	55	caruncular chorion intercaruncular chorion	+ +

*entire conceptus roughly 3 mm in length so question of caruncular or intercaruncular chorion (trophoblast) does not apply

**material obtained by flushing uterus so cannot be said if sections through chorion would be where it was overlying a caruncle or an intercaruncular area

antigen to antibody in the preincubation mixture was slightly higher than that employed by Chan et al (1978a) in radioimmunoassays using the same antiserum. Here, 0.1 ml of a 1:25 dilution of anti-oCS was exposed to 0.4 ml of oCS solution (5 g/ml phosphate buffered saline, pH 7.4). The near-adjacent sections were incubated with a 1:125 dilution of anti-oCS.

Results

Some of the experimental results have already been reported (Carnegie et al, 1980; see Appendix C). Included below are additional results not presented in the published paper.

With the exception of day 12, oCS is present in the chorionic layer at all stages examined. Repeat incubations of day 12 trophoblast using 2, 4 and 6 μ m sections revealed no fluorescent staining attributable to antibody binding. The same pattern of antibody distribution was observed at all of the remaining stages as was previously described for days 20 and 28 (Carnegie et al, 1980; see Appendix C), that is, fine lines of fluorescence in the cytoplasm of uninucleate cells encircling spherical bodies roughly 2 μ m in diameter (Figs. 70, 71, 73 and 74). Maximal antibody binding was observed at day 28, while at days 14 and 55, the fluorescent staining was noticeably reduced, indicating the presence of somewhat lower levels of hormone.

At the earlier stages, oCS distribution is quite uniform throughout the single layer of chorionic cells. A survey of day 14 tissue revealed varying intensities of fluorescence in the cytoplasm of every chorionic cell (Fig. 73); the inner lining of endodermal cells alone is negative (Fig. 74). The situation is little changed by day 16; only the occasional oCS-negative cell was encountered (Fig. 75). By day 28, oCS distribution has become quite localized. At this stage, rounded binucleate cells are easily distinguished from their columnar, uninucleate neighbours, not only on the basis of structural differences,

FIGURES 70, 71, 73 & 74

Pretreatment of sections with Schiff's reagent produces the red fluorescence seen in the colour micrographs. The green fluorescence is due to antibody binding.

FIGURE 69

Corresponding area to that in Fig. 70 (day 28 trophoblast). The labelling of the binucleate cells (bn) and crystals (c) in Figs. 69 and 70 permit the orientation of the reader. Acid fuchsin; phase contrast optics.

FIGURE 70

Binding of anti-oCS to day 28 trophoblastic tissue. The hormone is in the cytoplasm of uninucleate cells and surrounds small, spherical bodies. The binucleate cells (bn) are negative. c = crystal.

FIGURE 71

Binding of anti-oCS to day 28 trophoblastic tissue. The binucleate cell (bn) at the top is negative; antibody binding is confined to the cytoplasm of uninucleate trophoblastic cells.

FIGURE 72

Corresponding area to that in Fig. 71. The labelling of binucleate cells (bn) permits the orientation of the reader.

FIGURE 73

Binding of anti-oCS to the cytoplasm of day 14 trophoblastic tissue. Positive staining was observed in every trophoblastic cell at this time.

FIGURE 74

Binding of anti-oCS to the cytoplasm of a day 14 trophoblastic cell. The fluorescence surrounds spherical bodies roughly $2\mu\text{m}$ in diameter whose contents were not retained by glutaraldehyde fixation alone. Staining of osmium tetroxide post-fixed sections with sudan black B (see Ultrastructure and Histochemistry Results) indicates that these spherical bodies are lipid droplets. e = endodermal cell; n = nucleus.

COLOURED PICTURE

FIGURE 75

Portion of day 16 trophoblast showing positive staining for oCS (arrows) in most of the cells. A possible negative trophoblastic cell (pnc) is indicated.

FIGURE 76

Day 28 trophoblastic tissue with positive staining for oCS in the cytoplasm of uninucleate cells (arrows), but not in 2 binucleate cells (bnc). The weak fluorescence of the 2 binucleate cells is due to strong binding of Schiff's reagent.

FIGURE 77

Corresponding region to that in Fig. 76. The labelled binucleate cells (bnc) and arrows denoting localization of oCS permit the orientation of the reader. Acid fuchsin; phase contrast optics.

but also because they are consistently oCS-negative (Figs. 69-72, 76 and 77). Often, the cytoplasm of uninucleate cells in the immediate vicinity of the binucleates is oCS-positive (Figs. 70, 71 and 76), but antibody binding was never observed within the confines of the binucleate cell plasma membranes.

At day 28, only certain of the uninucleate chorionic cells are oCS-positive. By this time, the surface of the maternal caruncle is no longer smooth but is raised into numerous villi. The chorionic cells overlying the tips of the villi are taller than those reaching into the crypts and their swollen apical ends are filled with lipid droplets (Carnegie, unpublished observation based on sudan black B staining of day 30 osmium tetroxide postfixed, Spurr's-embedded placental tissue) interspersed with occasional crystals (Fig. 78). It is here where most of the antibody binding is localized (Figs. 69-72), the fluorescent lines emphasizing the boundaries of the droplets. Occasionally, 1 or 2 cells in other regions of the chorion, running parallel to the caruncular surface or extending partially into a crypt, are also oCS-containing, but, again the fluorescent staining is always surrounding lipid droplets (Figs. 79 and 80).

By day 55, the maternal caruncular surface and its overlying chorion have become a network of long, interlocking villi. Many of the uninucleate chorionic cells appear to be lipid-containing, but this could not be verified using sudan staining as suitably fixed material was not available. Numerous prominent crystals are present in some of these cells as well (Fig. 82). Binucleate cells were observed and, again, they are oCS-negative (Figs. 81

FIGURE 78

Section of day 28 placental tissue showing the junction between fetal trophoblast and maternal caruncular tissues (mat villus). Microvilli (mv) indicate the junction between the 2 cell layers. The trophoblastic cells at the tips of the villi are long and columnar (at) and filled with apical lipid droplets. The arrow indicates the approximate location of the tissue shown in Figs. 79 and 80.

FIGURE 79

One of the few examples of positive staining for oCS toward the crypts between the maternal villi. The arrow in Fig. 78 shows the type of region from which the tissue in this figure came. The nucleus (n), fetal red blood cell (f) (the fluorescence is due to strong binding of Schiff's reagent) and arrows permit the orientation of the reader.

FIGURE 80

Same area as that in Fig. 79. The nucleus (n), fetal red blood cell (f) and arrows permit the orientation of the reader. mv = microvilli between fetal and maternal layers.

30 μ m

FIGURE 81

Day 55 caruncular trophoblast incubated for oCS (green). The red fluorescence results from the pretreatment of sections with Schiff's reagent. c = crystal; bnc = binucleate cell.

FIGURE 82

Same region as that shown in Fig. 81. The binucleate cell (bnc) and crystals (c) permit the orientation of the reader. Acid fuchsin; phase contrast optics.

FIGURE 83

Day 55 intercaruncular trophoblast incubated for oCS (green). The red fluorescence results from the pretreatment of sections with Schiff's reagent. n = nucleus.

FIGURE 84

Same region as that shown in Fig. 83. The nucleus permits the orientation of the reader. Acid fuchsin; phase contrast optics.

COLOURED PICTURE

and 82). Overall fluorescent staining of the sections was considerably reduced over that at day 28. Groups of 2 to 4 cells contained small cytoplasmic regions of antibody binding and a survey of a large area of chorion indicated that, on the average, about 1 cell in 50 is oCS-containing.

The same situation exists in the intercaruncular chorion at day 55 (Figs. 83 and 84). Roughly the same proportion of fetal cells are oCS-positive and, again, the amount of fluorescent antibody bound by these cells was not very high, compared to day 28 caruncular chorion.

There was no antibody binding to sections of either day 14 or day 28 corpus luteum.

Preincubation of anti-oCS with oCS antigen did not appear to noticeably reduce antibody binding to either the day 28 or day 55 chorionic tissue.

Discussion

The immunolocalization of oCS at various stages of gestation has been attempted several times over the past few years by 3 separate groups of researchers (Table 4). Different localization results have been obtained by each group and their comparison is rendered somewhat more difficult in that the fixation and embedment techniques also varied (Table 4).

The initial studies focussed on the last trimester of pregnancy. Martal et al (1977) found that their anti-oCS preparation bound exclusively to the large, PAS-positive, mono- or binucleated cells of the fetal trophoblast. On the other hand, the antiserum to oCS used by Reddy and Watkins (1978b), in addition to labelling the PAS-positive binucleate cells, also bound to uninucleate trophoblastic cells which did not appear to be PAS-reactive. This study was subsequently extended to include some of the earlier stages of gestation (back to day 22) and further information obtained on the oCS-containing cells (Watkins and Reddy, 1980). They found that not all of the binucleate cells interacted with the anti-oCS serum and proposed the existence of 2 possible functional subgroups of this cell type; those involved in the production of the hormone oCS and those which produce some other type of secretory product. Regarding the uninucleate oCS-positive cells, they found them to be consistently present in the maternal syncytium, rather than fetal trophoblast, and postulated that they were either binucleate cells which had migrated across the feto-maternal junction or maternal

Table 4. Summary of Data - Immunolocalization of oCS

Reference and Stage of Gestation	Tissue Fixation and Embedment	Incubation Procedure*	Antiserum Specificity	Incubation Controls	Antibody Binding	Absence of Antibody Binding
Martal et al (1977) day 120	Bouin-Hollande for 48 h ↓ paraffin	1:40 dilution for 1 h**	Ouchterlony double immunodiffusion and preincubation of anti-oCS with various antigens	various control antisera normal rabbit serum	large mono- or binucleate PAS+ cells of chorion	PAS- cells
Reddy and Watkins (1978b) days 100-130	Bouin's for 2 wks ↓ Paraplast	1:10 dilution for 30 min at 37°C	preincubation of anti-oCS with oCS	normal rabbit serum rabbit anti-bGH	PAS+ binucleate cells uninucleate trophoblastic cells	
Carnegie et al (1980) days 20 and 28	glutaraldehyde 0.5 h on ice ↓ glycol methacrylate	1:10 dilution for 24 h at 4°C	Ouchterlony double immunodiffusion	normal rabbit serum	uninucleate trophoblastic cells	binucleate cells

Reference and Stage of Gestation	Tissue Fixation and Embedment	Incubation Procedure*	Antiserum Specificity	Incubation Controls	Antibody Binding	Absence of Antibody Binding
Watkins and Reddy (1980)	see Reddy and Watkins, 1978b	day 22: 1:10 dilution for 12 h at 37°C - all others as above	see Reddy and Watkins, 1978b	see Reddy and Watkins 1978b	some PAS+ binucleate cells	some binucleate cells***
days 22-145					uninucleate cells in maternal syncytium	

Carnegie days 12-55	see Carnegie et al, 1980	see Carnegie et al, 1980	see Carnegie et al, 1980	see Carnegie et al, 1980	uninucleate binucleate cells tropho-blastic cells day 12 from days 14- trophoblast	55
---------------------	--------------------------	--------------------------	--------------------------	--------------------------	--	----

*only the procedure for incubation with the first antiserum, anti-oCS, was reported here

**"Tissue sections were stained by sequential exposure to PAS/hematoxylin-safranin, after the reaction had been performed, in order to control the cytological specificity (dye affinity) of the immunoreactive structures." I am not sure what effect this step would have had on the specificity and interpretation of the staining procedures.

***proposed the existence of 2 classes of binucleate cells with respect to oCS production - those which produce oCS and those which do not

cells which were themselves capable of oCS synthesis. In contrast, the localization studies conducted in this laboratory have repeatedly found that the anti-oCS binding is confined entirely to the uninucleate trophoblastic cells and is not a property of the PAS-positive binucleate cells at days 16, 20, 28 or 55.

The production of oCS as early as day 14 of gestation has not been previously reported using either immunolocalization or radioimmunoassay (RIA) techniques. It has been shown, by RIA of tissue extracts, that day 16 blastocysts do contain oCS (Martal and Djiane, 1977a; Chan et al, unpublished observation). However, Watkins and Reddy (1980) have been unable to detect oCS using immunolocalization techniques earlier than day 22 in Bouin's-fixed, Paraplast-embedded tissue sections. Unfortunately they did not indicate which stages prior to day 22 were tested.

The immunolocalization of oCS is not the only hormone localization attempt to meet with such discrepancies in experimental results. A survey of the literature reporting the immunolocalization of human chorionic somatomammotropin (hCS) also indicates the presence of similar, although not so widely divergent, controversies (Table 5). There is general agreement that hCS is present in the syncytial cytoplasm of the fetal chorion (trophoblast) and that it is never found in the Langhans' cells (de Ikonickoff and Cedard, 1973; Osada, 1976; Dujardin et al, 1977). However, while one group reported an absence of anti-hCS binding to amniotic epithelium (de Ikonickoff and Cedard, 1973), another indicated that, not only did some of the amniotic epithelial cells appear to be hCS-containing, but also additional

Table 5. Summary of Data - Immunolocalization of hCS*

Reference and Stage of Gestation and Embedment	Tissue Fixation	Incubation Procedure	Antiserum Specificity	Incubation Controls	Antibody Binding	Absence of Antibody Binding
de Ikonicoff and Cedard (1973)	Bouin's or alcohol-formol-acetic acid to paraffin or fixed or non-fixed cryostat sections**	purge or 1:10 dilution - 45 min at room temp.	Ouchterlony double immunodiffusion immuno-electrophoresis	control tissue - rat cerebrum normal rabbit serum	syncytial cytoplasm of chorionic villi	Langhan's cells amniotic epithelium
de Ikonicoff (1973)	4% PF***-sucrose buffer 4% PF - cacodylate buffer 1.25% GA plus 1.25% PF****	anti-hCS for 1-24 h at 4°C fixation for 1 h at 4°C as before	same procedure as de Ikonicoff and Cedard, 1973	same controls as de Ikonicoff and Cedard, 1973	2 areas in syncytial cytoplasm: maternal side of syncytial microvilli and ER of fetal syncytium	Golgi, mitochondria and nuclear membranes of syncytial layer
term placentas (E.M. level)	↓ cryostat sections	standard prep. for E.M.				

Reference and Stage of Gestation	Tissue Fixation and Embedment	Incubation Procedure	Antiserum Specificity	Incubation Controls	Antibody Binding	Absence of Antibody Binding
Osada (1976)	95% ethanol - 24 h at 4°C ↓ paraffin		Ouchterlony double immunodiffusion immunoelectrophoresis		cytoplasm of syncytial layer	Langhan's cells
throughout gestation						
Dujardin et al (1977)	Bouin's Hollande for 8 days ↓ paraffin		preincubation of anti-hCS with hCS and hCG	control tissue - human pituitary normal rabbit serum	fetal syncytium giant cells cytotrophoblast amniotic epithelium	Langhan's cells
-6 wks to 40 wks						

*Localization is at light microscope level unless otherwise indicated. As with Table 4, only the incubation procedure for the first antiserum, the anti-hCS, is reported here.

**The same localization results were obtained with all of the tissue treatments.

*** PF = paraformaldehyde; GA = glutaraldehyde

****Fixation was for 30 min to 24 h and followed by incubation for hCS and then postfixation and embedment.

fetal chorionic cells, specifically the multinucleated giant cells of the basal plate and the chorionic cytotrophoblast stained positively with the anti-hCS serum (Dujardin et al, 1977).

These differences in experimental results raise a number of questions which apply equally well to the localization of either hCS or oCS. A variety of tissue fixation and embedment procedures were employed leading one to speculate on the degree of preservation of hormone antigenicity throughout these treatments. In the case of hCS, this potential problem was anticipated by de Ikonicoff and Cedard (1973) who compared localization results in fixed, paraffin-embedded tissue with those obtained using fixed or nonfixed cryostat sections and found them to be independent of tissue handling. In this study, it became evident that tissue fixed for 0.5 h in glutaraldehyde had to be maintained at a low temperature throughout all subsequent treatments, including embedment, if one was to get antibody binding (Carnegie et al, 1980; see Appendix C). However, the longer fixation procedures employed by Martal et al (1977) and by Reddy and Watkins (1978b; 1980) may well have provided enough extra protection for the oCS antigenic sites that they were able to withstand the higher temperature required for paraffin embedment.

Related to this is the question of per cent of antigen preservation. Different fixation techniques may inactivate varying proportions of the antigen population; with the possible result that cell types containing low levels of a given antigen would appear negative under one fixation regime and positive under another. Apparently, some sort of agreement must be reached

between research groups as to a means of obtaining a high degree of antigenic activity along with good structural preservation for a given antigen so that experimental results can be compared with as many experimental procedures as possible in common.

Assuming that the hCS or oCS antigenic sites had been adequately preserved in all of the tissue treatments employed, the only other factor which could account for the variations in localization results would be differences in antibody specificity. Some attempts were made to establish the specificities of the antisera employed, such as Ouchterlony immunodiffusion, pre-incubation of the antiserum with appropriate antigens and immunoelectrophoresis (Tables 4 and 5). However, not all of these controls were conducted by each group of researchers and studies involving the localization of other proteins have suggested that they may not be sufficient to rigorously determine antigen specificity anyway (see Appendix B for a complete treatment of this subject). More research is required before those criteria which unquestioningly establish antigen specificity can be worked out and a set of guidelines controlling immunolocalization experiments can be prepared.

According to the information presented in Appendix B, the specificity of the oCS antiserum used in this series of experiments has not been clearly established. Certain facts suggest that it is indeed oCS which is being localized. As mentioned before, RIA experiments have indicated that oCS is present in the day 16 blastocyst (Martal and Djiane, 1977a; Chan et al, unpublished observation). The sensitivity of this immunolocalization method

has not been determined; however it is possible that it is able to detect lower levels of hormone than RIA and is hence capable of indicating the presence of oCS in day 14 trophoblast whose concentration is below that currently measurable by RIA. That no antibody binding to day 12 trophoblast was obtained suggests that this is not the localization of an embryonic protein present in trophoblastic tissue throughout gestation. Finally, Ouchterlony immunodiffusion tests have indicated that the antiserum does indeed interact with purified oCS (Carnegie et al, 1980; see Appendix C).

The results of the preincubation control experiments suggest, at first glance, that it is not the hormone oCS which was localized in this study. However, a closer look reveals that the experiments were not conducted with the correct amount of antigen. The proportion of antigen to antibody in the preincubation solutions (2 μ g in 0.1 ml of a 1:25 dilution of anti-oCS) was what was found to be suitable for good standard curve production by radioimmunoassay (Chan et al, 1978a). But, as explained in Appendix B, in preincubation experiments, antigen must be added in large excess to fill all of the antigen-binding sites on the antibody molecules and to displace the binding equilibrium away from tissue-bound and toward soluble antigen. For example, Zimmerman et al (1974) used 200 times as much antigen preincubated with the same dilution of antiserum (1 μ g Gn-RH in 0.25 ml of a 1:25 dilution of anti-Gn-RH). As discussed at greater length in Appendix B, preincubation with too little antigen can produce a variety of experimental results which are difficult to interpret ranging from little to no blockage of staining to even possible

staining enhancement (Zimmerman et al, 1974). Unfortunately, insufficient antigen was available to repeat the preincubation experiments with altered antigen to antibody ratios. The value of the preincubation control experiment, as it turns out, is being questioned as a rigorous test for antiserum specificity (see Appendix B).

The lack of anti-oCS binding to corpora lutea taken at days 14 and 28 of gestation is puzzling. Chan et al (1978b) have demonstrated significant binding of oCS to corpora lutea from both pregnant and nonpregnant ewes, so clearly this tissue has receptors for oCS. If oCS were luteotrophic, one would expect it to begin protecting the corpus luteum at about day 12 of gestation and to continue preserving luteal function up to at least day 50 (Denamur and Martinet, 1955). Three possible explanations exist for the results obtained here. oCS is not luteotrophic (at least not by binding to the corpus luteum; it could perhaps exert a luteotrophic effect in an indirect manner) and hence one is not getting anti-oCS binding to luteal tissue because no oCS is there. Secondly, oCS is binding to luteal tissue but the receptors are so dispersed that they are not detected by this method. Thirdly, oCS binds to luteal tissue, but in a localized manner and the few sections taken for study did not happen to traverse a region of oCS binding. This study was not pursued at length because the specificity of the antiserum could not be proven and so I cannot, at this time, indicate which of these explanations or what alternative explanations would account for these experimental results.

Although this research project did not concern itself with the determination of the role or roles of oCS in early ovine gestation, two closely-timed and possibly related events occurring in the blastocyst are worthy of mention. The time of first detection of oCS in the trophoblast (day 14) coincides with a period of lipid accumulation by this same tissue (days 12 to 16; see Ultrastructure and Histochemistry Results). Handwerger et al (1975; 1976), by monitoring plasma free fatty acid levels following oCS administration, have suggested that this hormone promotes fat storage. It may be that this lipid accumulation stimulus is directed toward the developing conceptus, as well as the mother, at a time when it needs a large supply of energy to fuel its rapid growth. The presence of oCS in close proximity to the lipid droplets makes this idea more plausible and also causes one to wonder if one is seeing a site of oCS production or one of oCS utilization. Clearly, further experiments, using antisera whose specificity cannot be challenged and possibly extending the localization to the electron microscopic level, are required before these questions can be answered.

List of Conclusions

Between days 12 and 16 of gestation, ovine trophoblastic cells accumulate a large amount of lipoidal material. This is at the time of rapid blastocyst growth and it is suggested that the lipid may serve as an energy source prior to the establishment of placental contact with the mother.

Lysosomes of varying appearances populate the trophoblastic cells between days 12 and 16 of pregnancy. In addition to cellular debris, they may also contain $0.1\mu\text{m}$ bodies with electron-dense cores (especially at day 12) or protein-containing crystals (days 14 and 16).

Roughly 10% of the trophoblastic cells found in days 12 to 16 blastocysts are in the process of dying. This appears to be a normal feature of blastocyst development but its role or cause has yet to be determined.

Anti-oCS serum binds to trophoblastic tissue taken between days 14 and 55 of gestation, but not to that taken at day 12. The antibody binding is confined to the cytoplasm of uninucleate trophoblastic cells and surrounds spherical bodies roughly $2\mu\text{m}$ in diameter which, in suitably-prepared tissue sections, have been shown to contain lipid.

The oCS localization results are in conflict with those of other researchers who have obtained antibody binding to binucleate trophoblastic cells. Such discrepancies in experimental results are, in all probability, due to a lack of good control experiments to establish antiserum specificity.

Appendix ASolutions and Methods used in Tissue Preservation and Staining1. Buffersa) Sorenson's Phosphate Buffer (Lillie, 1965)

To make 0.1 M phosphate buffer, pH 6.8, 0.1 M K_2HPO_4 and 0.1 M KH_2PO_4 were prepared and mixed in equal volumes.

b) Phosphate Buffered Saline

Two solutions, 0.1 M NaH_2PO_4 and 0.1 M Na_2HPO_4 , were prepared. They were combined in equal volumes and NaCl (9 g/l) was added. The pH was adjusted to 7.4 with 1 N NaOH.

2. Embedment Solutionsa) Glycol Methacrylate (GMA) (Ashford et al, 1972)

glycol methacrylate (Hartung Associates)	95 ml
polyethylene glycol (Fisher Scientific)	5 ml
azobisisobutyronitrile (Eastman Organic Chemicals)	0.15 g

The above were mixed for 30 min, and the resultant solution filtered and bubbled with N_2 gas (to remove excess O_2 due to air bubbles arising from mixing) prior to use.

b) Spurr's Resin (Spurr, 1969)

ERL (vinylcyclohexene dioxide - Polysciences, Inc.)	3 g
---	-----

DER (diglycidyl ether of polypropylene glycol - Polysciences, Inc.)	1.5 g
NSA (nonenylsuccinic anhydride - Polysciences, Inc.)	7.8 g
S-1 (dimethylaminoethanol - Polysciences, Inc.)	0.12 g

These 4 chemicals were combined in the order and weights given, mixing well after each addition. A fresh solution was prepared each time.

3. Staining Procedures

a) Uranyl Acetate (Dr. E. H. Newcomb, personal communication)

A solution of 2% uranyl acetate (Fisher Scientific) was prepared in double distilled water (0.2 g in 10 ml) and sonicated for 1 h. The solution was filtered through Whatman no. 50 paper and stored in a foil-covered flask at 4°C.

For staining, the uranyl acetate solution was brought to room temperature and the grids incubated, section side up, in drops on dental wax for 45 min. Rinsing was accomplished by dipping the grids quickly in filtered distilled water (roughly 20 times each in 3 changes of water) and allowing them to air dry.

b) Reynolds Lead Citrate (Reynolds, 1963; modified preparation - personal communication from Dr. A. Hardham)

Two stock solutions, lead nitrate (2.66 g/100 ml distilled water; Fisher Scientific) and sodium citrate (3.52 g/100 ml distilled water; Fisher Scientific) were prepared and stored at room temperature. Immediately before staining, 2.1 ml of each were combined and shaken well so that a precipitate was formed.

To this was added 0.8 ml of 1 M NaOH and the solution mixed well to dissolve the precipitate.

The grids were incubated in drops of the staining solution for 30 min at room temperature, rinsed briefly in filtered distilled water and allowed to air dry.

c) Paragon Stain

Mixture A was prepared by placing 0.365 g toluidine blue (BDH Chemicals) and 0.136 g basic fuchsin (Allied Chemical) in 50 ml of 30% ethanol. Mixture B was 2% aqueous sodium borate (Fisher Scientific). To 20 drops of A was added 3 drops B; the two were mixed thoroughly with a pasteur pipet and immediately transferred to the tissue sections through a Millipore filter. Staining was for 30 - 45 sec on a hot plate (90 - 100°C) and immediately followed by thorough rinsing in running water. The slides were mounted in immersion oil.

4. Histochemical Incubations

a) Acid Phosphatase (Barka and Anderson, 1962*)

Stock Solutions

Acetate Buffer (0.2 M) (Barka and Anderson, 1965)

Solution A - 0.4 M sodium acetate.

Solution B - 0.4 M acetic acid

To obtain acetate buffer, pH 5.0, 35.2 ml A was combined with 14.8 ml B.

Substrate Solution

100 mg naphthol AS-BI phosphate (Sigma Chemical Co.) was placed in 10 ml N,N-dimethyl-formamide (Sigma Chemical Co.). This solution was prepared freshly for each incubation.

Pararosanilin Solution

0.2 g. pararosanilin hydrochloride (Sigma Chemical Co.) was placed in a solution of 4 ml distilled water and 1 ml concentrated HCl. It was warmed gently, while stirring, until dissolved. The solution was then cooled and filtered. It was also prepared freshly for each incubation.

Sodium Nitrite Solution

This solution, which was prepared freshly each week, consisted of 4% sodium nitrite in distilled water.

The working solution was prepared immediately before incubation. First, 8.5 ml of the acetate buffer, pH 5.0, was combined with 8.5 ml distilled water and to this was added 1 ml of the substrate solution. Then, 0.8 ml pararosanilin solution was added to 0.8 ml sodium nitrite, mixed and then added to the buffered substrate solution. The pH was adjusted to 5.0 with 1 N NaOH.

A control incubation solution was prepared each time, as above, except that the naphthol AS-BI phosphate was omitted.

Incubation was accomplished by flooding slides with either the working or the control solutions and placing them in a moist chamber for 1.5 h at room temperature. They were rinsed thoroughly in running water, air dried and mounted in immersion oil for optical microscopy.

*The Michaelis veronal acetate buffer used by Barka and Anderson (1962) was changed to 0.2 M acetate buffer (Burstone, 1958).

b) Naphthol Yellow S (Deitch, 1955)

Sections were stained in 1% naphthol yellow S (Allied Chemical) in 1% acetic acid for 15 min at room temperature. They were differentiated overnight in 1% acetic acid, rinsed in running water for 5 min, air dried and mounted in immersion oil.

c) Mercury-Bromphenol Blue (Barka and Anderson, 1965)

Sections were stained for 15 min at room temperature in a solution of 10 g mercuric chloride and 100 mg bromphenol blue (Fisher Scientific) in 100 ml of 95% ethanol. They were washed for 20 min in 3 changes of 0.5% acetic acid, rinsed in water and then incubated in 0.01 M phosphate buffer, pH 7 for 3 min. They were once more rinsed in water, air dried and mounted in immersion oil.

d) Aniline Blue Black (Fisher, 1968)

Sections were stained in 1% aniline blue black (naphthol blue black; Polysciences Inc.) in 7% acetic acid for 10 min at 60°C. They were then dipped briefly in 7% acetic acid, air dried and mounted in immersion oil.

e) Protease Digestion of Tissue Sections (Dr. M. Smith, personal communication)

To 100 ml distilled water were added 0.75 g glycine and 0.074 g CaCl_2 and the pH adjusted to 7.5 with 0.1 N NaOH. To 5 ml of this solution, on ice, 25 mg protease (pronase AS; Sigma Chemical Co.) was added. The slides were flooded with the solution

and incubated in a moist chamber which was sealed in parafilm and wrapped in foil for 24 h or 48 h at 37°C. The slides were rinsed gently in distilled water by puddling (Carnegie *et al*, 1980, see Appendix C) and mounted in immersion oil. Control slides were incubated in the glycine buffer alone (no protease).

f) Papain Digestion of Tissue Sections (Glenner and Lillie, 1959)

To 10 ml phosphate buffer, pH 7.0 (39.0 ml 0.2 M NaH_2PO_4 plus 61.0 ml 0.2 M Na_2HPO_4) was added 10 mg papain (Sigma Chemical Co.). Incubation was for 48 h at 37°C. Control incubations omitted the inclusion of papain in the buffered solution. All slides were mounted in immersion oil.

g) Sudan Black B

Pretreatment of Sections to remove Osmium Tetroxide (Lewis and Knight, 1977)

Sections were treated with 10% hydrogen peroxide (110 volumes; BDH Chemicals) for 30 min at room temperature and rinsed in running water prior to incubation with sudan black B.

Staining with Sudan Black B (Pearse, 1972)

The sections were dipped in 70% ethanol and then incubated in a saturated solution of sudan black B (Edward Gurr, Ltd) in 70% ethanol. (The staining solution was filtered prior to use). Incubation was for 16 h at 60°C. This was followed by a brief rinse in 70% ethanol, a thorough washing in running water and mounting in immersion oil.

h) Periodic Acid-Schiff's Reaction (Feder and O'Brien, 1968)

Sections were incubated in the following solutions:

- 1) 2,4 dinitrophenyl hydrazine (Eastman Organic Chemicals) -

a saturated solution in 15% v/v acetic acid, freshly prepared and filtered prior to use - 10 min

2) running water rinse - 30 min

3) 1% periodic acid (J. T. Baker Chemical Co.) - 10 min

4) Schiff's reagent (Fisher Scientific) - 10 min

5) 3 changes of 0.5% $\text{Na}_2\text{S}_2\text{O}_5$ (Fisher Scientific), 2 min each

6) running water rinse - 10 min

They were mounted in immersion oil for optical microscopy.

Appendix B

Immunofluorescent Techniques - A Critical Appraisal

1. The Technique Itself

The immunofluorescent localization of tissue antigens can be carried out using either a single or a double antibody method (Diagram 3). In the former, a fluorescent marker, usually fluorescein isothiocyanate (FITC) or rhodamine is attached to an antibody produced to a given antigen A. The labelled anti-A is applied to tissue sections, incubated for a given time interval and then all unbound label is removed using thorough washing procedures. In the latter technique, also known as the sandwich method, anti-A is not labelled. (Consider, here, that anti-A was raised in a rabbit.) Following the application of anti-A to the tissue sections, they are rinsed and then incubated with a second antibody, which does carry the fluorescent tag. This would be an antiserum to rabbit proteins in general raised in another species, for example, FITC goat anti-rabbit γ globulin.

The double antibody technique, first derived by Coons et al (1955), has 2 advantages. First, it provides greater sensitivity of localization. Each antigen has more than one antibody-binding site (Petrusz et al, 1976). Therefore, when 2 layers of antibodies are employed, more labelled antibody molecules are bound per molecule of antigen than if only one were used (Diagram 3). Secondly, the components are less expensive and easier to obtain,

Diagram 3. A comparison of sensitivity using single and double antibody methods.

Single antibody method

Double antibody method

since one does not have to attach a fluorescent marker to each different antibody employed, but can simply use a commercially-available general antiserum to whatever species was used for antibody production.

The control experiments described in the next section relate primarily to the double antibody technique, as that was the one chosen for this project. However, many of them could be adapted to the single antibody method and so this appendix should be considered a critique of immunofluorescent localization methods, in general.

2. Critique

The literature is filled with papers reporting the localization of antigens such as actin, tubulin or various pituitary hormones using immunofluorescence or immunoelectronmicroscopic techniques, but the number of papers also published debating the validity of the results obtained using these methods (eg. Petrusz et al, 1976; Petrusz et al, 1977; Swaab et al, 1977; Forer, 1978; Hutson et al, 1979) testify to the technical problems still requiring solution. Swaab et al summarized the primary difficulty troubling those using these techniques in their 1977 letter to the editor of J. Histochem. Cytochem. - "Can specificity ever be proved in immunocytochemical staining?".

Antisera which have been prepared for radioimmunoassay are often not adequate for specific antigen localization on tissue sections. Antigens are obtained from biological material and for

9

this reason can never be considered 100% pure (Swaab et al, 1977). Hence, injection of an antigen preparation into a rabbit will give rise to a population of antibody molecules produced both to different regions of the antigen molecule under study and to whatever other proteins contaminate the preparation.

Radioimmunoassay is a technique used to measure the concentration of an antigen in which the unknown amount of antigen in the solution competes for antibody binding with a known concentration of radioactively-labelled antigen molecules. Hence, specificity is largely controlled by the labelled antigen used (Petrusz et al, 1976). For example, consider that a given antiserum is composed of 2 populations of antibodies, those that can bind to antigen A and those that can bind to antigen B (Diagram 4). If antigen A is the one to be measured in the assay, labelled A will be added to the solution to compete for antibody binding with the unknown amount of unlabelled A. That the antibody can also bind B is not a problem, even if B is also present in the solution, because only A can displace labelled A from the antibody and so be measured.

In immunofluorescence, however, no such internal control over specificity is present. Again with the same antibody preparation and with the interest focussed on antigen A, binding to tissue sections will occur under 3 possible conditions; if A is present, if B is present or if A and B are present. Hence, it would be very easy, using an antibody preparation considered specific by radioimmunoassay standards, to localize an antigen

Diagram 4. A comparison of antiserum specificity in radioimmunoassay and immunolocalization.

a) Radioimmunoassay

b) Immunolocalization

c) Pre-incubation control

(Petrusz et al, 1976, p. 1111)

not even present in the tissue section! This possibility has led to the introduction of extra control experiments designed to determine if the antiserum is composed of a single population of antibody molecules or if additional purification procedures are required.

One such control which is widely used is the preincubation of the antiserum with its homologous antigen, that is, the antigen to which the antibody in question was raised (eg. Moriarty, 1975). The theory behind this control is that the presence of excess antigen will fill all of the antigen-binding sites on the antibody molecules so that the subsequent application of the antibody to the tissue sections, if it is specific for that antigen, will result in no binding to and hence no fluorescent labelling of the section. If the antiserum does contain other factors which are nonspecifically interacting with some tissue component, this interaction will still take place and can now be recognized as nonspecific binding (Diagram 4).

This control has been taken one step further and, to some antigens, monospecific antibodies have been produced (eg. Fuller et al, 1975; Baumgartner et al, 1978; Schliwa et al, 1978). Here, the antigen is coupled to Sepharose or polyacrylamide-agarose beads or to ultragel and this complex is packed into a column. Under suitable conditions, application of antiserum allows the antibody molecules to bind to the column while all other constituents pass through. Further washing of the column with buffer ensures the removal of all contaminants. The replacement

of the neutral ~~buffer~~ with one more acidic removes the bound antibody and subsequent concentration techniques yield a solution containing monospecific antibody which can be used to localize a single antigen in suitably prepared tissue sections.

Unfortunately, both the preincubation control and the production of monospecific antibodies face the same pitfalls. As mentioned before, antigens are never 100% pure and this control can only be as good as the purity of the antigen employed. Any antibodies in the antisera produced to impurities in the antigen preparation would also be removed by the preincubation procedure and would not be recognized as nonspecific binding. Furthermore, the "monospecific" antibody produced by column purification, again, would still be composed of a mixture of whatever antibodies were produced in reaction to the "purified" antigen.

There are other drawbacks to the use of the preincubation control. Often, a large excess of antigen is required to get complete blockage of staining and this is not always available. For example, in some work by Zimmerman et al (1974) on the localization of gonadotropin-releasing hormone (Gn-RH) in the mouse hypothalamus, the addition of 1 mg of Gn-RH to 0.25 ml of a 1:25 dilution of anti-Gn-RH (the dilution used for antigen localization on the tissue sections) only reduced staining by 80%. It is possible that, in some instances, the configuration of the antigen in the tissue section gives it a greater affinity for antibody binding than that of the antigen in solution (Swaab and Pool, 1975). The high concentration of antigen in the

preincubation solution would then displace the binding equilibrium away from the tissue-bound antigen and toward the soluble one.

An interesting observation made by Zimmerman et al (1974) is that preincubation with small amounts of antigen (10 μ g Gn-RH added to 0.5 ml of a 1:50 dilution of anti-Gn-RH) enhanced antibody binding to the tissue sections. A possible explanation for this can be given. Antibody molecules usually have more than one binding site for a given antigen (Petrusz et al, 1976). If insufficient soluble antigen is present to fill all the antigen-binding sites on the antibody molecules, interactions between tissue-bound antigen, antibody, soluble antigen and then more antibody molecules could give rise to large complexes at the site of a single antigen molecule. These complexes would then bind more molecules of FITC-labelled second antibody, giving rise to enhanced, rather than decreased, staining.

Hence, if not enough antigen is available to preincubate with a large excess, preincubation with moderate amounts of antigen could lead to either enhancement or only partial abolition of staining, results whose precise interpretation would be difficult to determine.

Swaab et al (1977) also raised the problem of cross-reaction, specifically as it related to their efforts to localize the structurally similar pituitary hormones oxytocin and vasopressin. Having found that preincubation of either anti-oxytocin or anti-vasopressin with its homologous antigen did not completely block fluorescent staining of tissue sections, Swaab and Pool (1975)

coupled the hormones to agarose beads which were then attached to glass slides so as to better study the binding characteristics of their antibody preparations.

They found weak cross-reactivity between anti-oxytocin and vasopressin and considerably more between anti-vasopressin and oxytocin. They interpreted this to mean that some antibodies had been produced, particularly in the case of anti-vasopressin, which were capable of interacting with either antigen; that is, they must have been produced to a portion of the polypeptide chain that is common to both hormones. They were able to remove the nonspecific antibody molecules by incubating the antiserum repeatedly with agarose bead-coupled heterologous antigen (that is, not the antigen to which the antiserum was raised; but the one which was also capable of cross-reacting with the antiserum) and removing the antibody-antigen-bead complex by centrifugation. This technique provides a very useful way of purifying and monitoring the purification of antibody preparations, provided, as pointed out by Swaab et al (1977), one knows what possible cross-reacting antigens could be present in the tissue sections.

One means of determining the number of components in a tissue section capable of cross-reacting with an antiserum is to investigate precipitation reactions between crude tissue extracts and antisera preparations by double immunodiffusion and immunoelectrophoresis, as described by Fujiwara and Pollard (1976) for their antomyosin preparations. In the case of double immunodiffusion, the antiserum is placed in the central well of an

agarose gel and the various test antigen solutions in a surrounding circle of wells. Fujiwara and Pollard (1976) found that only a single component in the crude extracts of platelets or of HeLa cells cross-reacted with the anti-myosin. Immuno-electrophoresis, in which the tissue extract is fractionated according to the electrophoretic mobilities of the proteinaceous components prior to antibody incubation yielded a further bit of information. The single tissue component cross-reacting with the antiserum has the same electrophoretic mobility as myosin.

Forer (1978), while recognizing that the use of this technique makes a significant contribution toward the characterization of antisera, pointed out 2 weaknesses. The preparation of the tissue extracts involved centrifugation to remove insoluble cellular material which was, therefore, not tested for cross-reaction with the antiserum. Olden and Yamada (1977) avoided this problem by investigating antibody binding to all of the components of a cell which were separated by whole cell gel electrophoresis.

The second, possibly more serious, weakness is that the tests for specificity employed fresh tissue while the actual localization of myosin was performed on fixed material. Hence, interactions between denatured (fixed) proteins and the antiserum were not taken into account and evidence does exist in the literature that some antibodies recognize denatured rather than native proteins. Mabuchi and Okuno (1977) prepared an antiserum to starfish egg myosin and found, by double immunodiffusion of agarose gels, that it also cross-reacted with myosins from

starfish tube feet, bovine platelets or rabbit skeletal muscle in the presence of sodium dodecylsulfate (SDS) but not in the presence of 0.6 M KCl. They suggested that SDS denaturation released antigenic sites previously inaccessible to the antibody molecules because they are normally located on the inside of the large protein chain.

Nishino and Watanabe (1977) found that their antiserum to 14S dynein from Tetrahymena cilia would also cross-react with dyneins from other species and with rabbit skeletal muscle actin, but again only if these proteins were denatured rather than in their native configurations. The possibility was once more raised that denaturation unmask common antigenic determinants. These results suggest that one has to become more aware of the effects of purification prior to antibody production on the structure of the antigen molecule and interpret experimental results always keeping in mind that the antigen used to produce the antiserum has not been treated in the same manner as the antigen to be localized in the tissue sections.

Summarizing, what can one do with immunolocalization techniques at this time? I think one has to accept that it is very difficult right now to produce and prove the existence of an antibody solution capable of interacting with one and only one tissue component. However, the use of the techniques of Swaab et al (1977) should allow the elucidation of much more information about a given antiserum and, if one has any knowledge of what cross-reacting components may be present, their removal in a fairly

straightforward manner. The combination of immunolocalization methods with other techniques such as tissue extraction and radioimmunoassay to demonstrate the presence of the antigen would be very useful. Hutson et al (1979) recommend the combining of immunolocalization experiments with physiological procedures, such as organ removal, which would help establish antiserum specificity. Their example was the localization of FSH (produced by the anterior pituitary) in the Sertoli cells of the rat testis. Their obtainment of the same level of antibody binding to sections of testis taken from rats hypophysectomized (pituitary removed) 60 days prior to sacrifice as to testis sections from intact animals revealed that the antiserum was also capable of binding to another protein which was not FSH. Unfortunately this type of control experiment could not be used in this project, since one is attempting to localize a hormone in the tissue in which it is being produced. But it appears to be a very useful control experiment which should be included whenever possible. Finally, the binding of the antigen to agarose beads (Swaab and Pool, 1975) which were then subjected to the same fixation and embedment procedures as the tissue would show that the antigenic sites could survive these treatments and still be localized.

This is a technique which can yield much valuable information and should not be abandoned. Hopefully, the future will provide a universally-accepted means of purifying and characterizing antisera. Until that time, one must simply interpret the results with caution and, where possible, combine them with other complementary experimental procedures.

PREVIOUSLY COPYRIGHTED MATERIAL
IN APPENDIX C, NOT MICROFILMED.

MAY BE OBTAINED FROM:

The Journal of Histochemistry
and Cytochemistry.
Copyright © 1980 by The Histochemical
Society, Inc.

"EMBEDMENT IN GLYCOL METHACRYLATE AT LOW
TEMPERATURE ALLOWS IMMUNOFLUORESCENT
LOCALIZATION OF A LABILE TISSUE PROTEIN"

Authors:

J.A. Carnegie
M.E. McCully
H.A. Robertson

Department of Biology
Carleton University
Ottawa, Ontario, CANADA.
K1S 5B6

and

Animal Research Institute
Ottawa, Ontario, CANADA.
K1A 0C6

References

- Abraham, R., Hendy, R., Dougherty, W.J., Fulfs, J.C., and L. Goldberg, 1970: Participation of lysosomes in early implantation in the rabbit. *Expt'l and Molec. Path.* 13, 329-345.
- Amoroso, E.C., 1952: Placentation. *In* Marshall's Physiology of Reproduction. (A.S. Parkes, ed.) Great Britain: Longman's, Green and Co.
- Ashford, A.E., Allaway, W.G., and M.E. McCully, 1972: Low temperature embedding in glycol methacrylate for enzyme histochemistry in plant and animal tissues. *J. Histochem. Cytochem.* 20, 986-990.
- Ashford, A.E., and M.E. McCully, 1970: Localization of naphthol AS-BI phosphatase activity in lateral and main root meristems of pea and corn. *Protoplasma* 70, 441-456.
- Baker, J.R., 1958: Principles of Biological Microtechnique. London; Methuen.
- Barka, T., and P.J. Anderson, 1962: Histochemical methods for acid phosphatase using hexazonium pararosanilin as coupler. *J. Histochem. Cytochem.* 10, 741-753.
- Barka, T., and P.J. Anderson, 1965: Histochemistry, Theory, Practice and Bibliography. U.S.A.: Harper and Row Publishers, Inc.
- Baumgartner, B., Tokuyasu, K., and M.J. Chrispeels, 1978: Localization of vicilin peptidohydrolase in the cotyledons of mung bean seedlings by immunofluorescence microscopy. *J. Cell Biol.* 79, 10-19.
- Bindon, B.M., 1971: Systematic study of preimplantation stages of pregnancy in the sheep. *Aust. J. Biol. Sci.* 24, 131-147.
- Bjorkman, N.H., 1969: Light and electron microscopic studies on cellular alterations in the normal bovine placentome. *Anat. Rec.* 163, 17-30.
- Bjorkman, N.H., and G. Bloom, 1957: On the fine structure of the foetal-maternal junction in the bovine placentome. *Z. Zellforsch.* 45, 649-659.
- Bloom, W., and D.W. Fawcett, 1968: A Textbook of Histology. U.S.A.: W.B. Saunders Company.
- Bonnet, R., 1882: Die Uterinmilch und ihre Bedeutung für die Frucht. *In* Beiträge zur Biologie. (Th. L.W. Von Bischoff, ed.) Stuttgart: Verlag Der J.G. Cotta'shen Buchhandlung.

Bonnet, R., 1888: Präparate und Zeichnungen zur Entwicklungsgeschichte des Schafes. Arch. Anat. Physiol., anat. Abt., 1-106.

Boshier, D.P., 1969: A histological and histochemical examination of implantation and early placental formation in sheep. J. Reprod. Fert. 19, 51-61.

Boshier, D.P., and H. Holloway, 1977: The sheep trophoblast and placental function: an ultrastructural study. J. Anat. 124, 287-298.

Boyd, J.D., and W.J. Hamilton, 1952: Cleavage, early development and implantation of the egg. In Marshall's Physiology of Reproduction. (A.S. Parkes, ed.) Great Britain: Longman's, Green and Co.

Bryden, M.M., Evans, H.E., and W. Binns, 1972: Embryology of the sheep: extraembryonic membranes and the development of body form. J. Morph. 138, 169-186.

Burstone, M.S., 1958: Histochemical demonstration of acid phosphatases with naphthol AS-phosphates. J. Nat. Cancer Inst. 21, 523-539.

Buttle, H.L., Forsyth, I.A. and G.S. Knaggs, 1972: Plasma prolactin measured by radioimmunoassay and bioassay in pregnant and lactating goats and the occurrence of a placental lactogen. J. Endocr. 53, 483-491.

Calarco, P.G., and E.H. Brown, 1969: An ultrastructural and cytological study of preimplantation development of the mouse. J. Exp. Zool. 171, 253-284.

Calarco, P.G., and A. McLaren, 1976: Ultrastructural observations of preimplantation stages of the sheep. J. Embryol. exp. Morph. 36, 609-622.

Calarco, P.G., and D. Szollosi, 1973: Intracisternal A particles in ova and preimplantation stages of the mouse. Nature, New Biology 243, 91-93.

Cerruti, R.A. and W.R. Lyons, 1960: Mammogenic activities of the mid-gestational mouse placenta. Endocrinology 67, 884-887.

Chan, J.S.D., Robertson, H.A., and H.G. Friesen, 1976: The purification and characterization of ovine placental lactogen. Endocrinology 98, 65-76.

Chan, J.S.D., Robertson, H.A., and H.G. Friesen, 1978a: Maternal and fetal concentrations of ovine placental lactogen measured by radioimmunoassay. Endocrinology 102, 1606-1613.

Chan, J.S.D., Robertson, H.A., and H.G. Friesen, 1978b: Distribution of binding sites for ovine placental lactogen in the sheep. *Endocrinology* 102, 632-640.

Chang, M.C., 1962: Development of bovine blastocyst with a note on implantation. *Anat. Rec.* 113, 143-162.

Chang, M.C., and L.E.A. Rowson, 1965: Fertilization and early development of Dorset Horn sheep in the spring and summer. *Anat. Rec.* 152, 303-316.

Christie, G.A., 1967: Histochemistry of implantation in the rabbit. *Histochemie* 9, 13-29.

Clark, R.T., 1934: Studies on the physiology of reproduction in the sheep: II. The cleavage stages of the ovum. *Anat. Rec.* 60, 135-159.

Coons, A.H., Leduc, E.H., and J.M. Connolly, 1955: Studies on antibody production. I. A method for the histochemical demonstration of specific antibody and its application to a study of the hyper-immune rabbit. *J. Exp. Med.* 102, 49-60.

Daniel, J.C.Jr., and J.R. Kennedy, 1978: Crystalline inclusion bodies in rabbit embryos. *J. Embryol. exp. Morph.* 44, 31-43.

Davies, J., 1952: Correlated anatomical and histochemical studies on the mesonephros and placenta of the sheep. *Amer. J. Anat.* 91, 263-299.

Davies, J., and H. Hesseldahl, 1971: Comparative embryology of mammalian blastocysts. In *The Biology of the Blastocyst*. (R.J. Blandau, ed.) U.S.A.: University of Chicago Press.

Davies, J., and W.A. Wimsatt, 1966: Observation on the fine structure of the sheep placenta. *Acta. Anat.* 65, 182-223.

de Ikonikoff, L.K., and L. Cedard, 1973: Localization of human chorionic gonadotropic and somatomammotropic hormones by the peroxidase immunohistochemical method in villi and amniotic epithelium of human placentas (from six weeks to term). *Am. J. Obstet. Gynecol.* 116, 1124-1132.

Deitch, A.D., 1955: Microspectrophotometric study of the binding of the anionic dye, Naphthol Yellow S, by tissue sections and by purified proteins. *Lab Invest.* 4, 324-351.

Denamur, R., and J. Martinet, 1955: Effets de l'ovariectomie chez la brebis pendant la gestation. *C. r. Séanc. soc. Biol.* 149, 2105-2107.

Dujardin, M., Robyn, C., and P. Wilkin, 1977: Mise en évidence immuno-histoenzymologique de l'hormone chorionique somatomammotrope (hCS) au niveau des divers constituants cellulaires du placenta humain normal. *Biol. Cell.* 30, 151-154.

Eddy, E.M., and J. M. Clark, 1975: Electron microscopic study of migrating primordial germ cells in the rat. *In* *Electron Microscopic Concepts of Secretion*. (M. Hess, ed.) U.S.A.: John Wiley and Sons.

Eisen, H.N., 1974: *Immunology. An Introduction to Molecular and Cellular Principles of the Immune Response*. U.S.A.: Harper and Row Publishers, Inc.

El-Shershaby, A.M., and J.R. Hinchliffe, 1974: Cell redundancy in the zona-intact pre-implantation mouse blastocyst: a light and electron microscope study of dead cells and their fate. *J. Embryol. exp. Morph.* 31, 643-654.

Enders, A.C., 1962: The structure of the armadillo blastocyst. *J. Anat.* 96, 39-48.

Enders, A.C., 1971: The fine structure of the blastocyst. *In* *The biology of the Blastocyst*. (R.J. Blandau, ed.) U.S.A.: University of Chicago Press.

Evans, H.E., and W.O. Sack, 1973: Prenatal development of domestic and laboratory mammals: growth curves, external features and selected references. *Anat., Histol., Embryol.* 2, 11-45.

Fawcett, D.W., 1967: *The Cell. An Atlas of Fine Structure*. U.S.A.: W.B. Saunders Company.

Feder, N., and T.P. O'Brien, 1968: Plant microtechnique: some principles and new methods. *Amer. J. Bot.* 55, 123-142.

Fellows, R.E., Bolander, F.F., Hurley, T.W., and S. Handwerker, 1976: Isolation and characterization of bovine and ovine placental lactogen. *In* *Growth Hormone and Related Peptides* (A. Pecile and E. Muller, ed.) Holland: Excerpta Medica Foundation.

Fisher, D.B., 1968: Protein staining of ribboned epon sections for light microscopy. *Histochemie* 16, 92-96.

Fléchon, J.-E., 1978: Morphological aspects of embryonic disc at the time of its appearance in the blastocyst of farm animals. *Scanning Electron Microscopy* 2, 541-548.

Flint, A.P.F., Henville, A., and W.B. Christie, 1979: Presence of placental lactogen in bovine conceptuses before attachment. *J. Reprod. Fert.* 56, 305-308.

Förer, A., 1978: Chromosome movements during cell-division: possible involvement of actin filaments. *In* Nuclear Division in the Fungi. (I.B. Heath, ed.) U.S.A.: Academic Press, Inc.

Forsyth, I.A., 1972: Use of a rabbit mammary gland organ culture system to detect lactogenic activity in blood. *In* Lactogenic Hormones, Ciba Fdn. Symp. no. 130. (G. Wolstenholme and J. Knight, ed.) Great Britain: Churchill Livingstone.

Forsyth, I.A., 1973: Secretion of a prolactin-like hormone by the placenta in ruminants. *In* Le Corps Jaune (R. Denamur and A. Netter, ed.) France.

Friesen, H.G., 1965: Purification of a placental factor with immunological and chemical similarity to human growth hormone. *Endocrinology* 76, 369-381.

Fujiwara, K., and T.D. Pollard, 1976: Fluorescent antibody localization of myosin in the cytoplasm, cleavage furrow, and mitotic spindle of human cells. *J. Cell Biol.* 71, 848-875.

Fuller, G.M., Brinkley, B.R., and J.M. Boughter, 1975: Immunofluorescence of mitotic spindles by using monospecific antibody against bovine brain tubulin. *Science* 187, 948-950.

Glenner, G.G., and R.D. Lillie, 1959: Pepsin release of guinea pig enterochromaffin substance. *J. Histochem. Cytochem.* 7, 204.

Green, W.W., and L.M. Winters, 1945: Prenatal development of the sheep. *U. of Minn. Agric. Expt. St. Tech. Bull.* 169, 1-35.

Greenstein, J.S., Murray, R.W., and R.C. Foley, 1958: Observations on the morphogenesis and histochemistry of the bovine preattachment placenta between 16 and 33 days of gestation. *Anat. Rec.* 132, 321-341.

Guillomot, M., 1979: Etude des modifications ultrastructurales et cytochimiques de la surface epitheliale uterine et du blastocyste de brebis au cours de l'implantation. Thèse. France: U. Paris P. et M. Curie.

Guillomot, M., Fléchon, J.-E., Wintenberger-Torrès, S. and P. Guay, 1980: Uterine and trophoblastic cell surface changes during implantation in the ewe and in the cow. Abstract presented to Southern Ontario Reproductive Biology Workshop, Ottawa, Canada.

Hadek, R. and H. Swift, 1960: A crystalloid inclusion in the rabbit blastocyst. *J. Biophys. Biochem. Cytol.* 8, 836-839.

Hall, F.J., Horne, R.W., and J.S. Perry, 1965: Electron microscope observations on the structure of cytoplasmic filaments in the pig blastocyst. *J. Roy. Microscop. Soc.* 84, 143-154.

Handwerger, S., Maurer, W.F., Barret, J., Hurley, T.W., and R.E. Fellows, 1974: Evidence for homology between ovine and human placental lactogens. *End. Res. Commun.* 1, 403-413.

Handwerger, S., Maurer, W.F., Crenshaw, M.C., Hurley, T.W., Barret, J., and R.E. Fellows, 1975: Development of the sheep as an animal model to study placental lactogen physiology. *J. Ped.* 87, 1139-1143.

Handwerger, S., Fellows, R.E., Crenshaw, M.C., Hurley, T.W., Barret, J., and W.F. Maurer, 1976: Ovine placental lactogen: acute effects on intermediary metabolism in pregnant and non-pregnant sheep. *J. Endocr.* 69, 133-137.

Handwerger, S., Crenshaw, M.C., Maurer, W.F., Barret, J., Hurley, T.W., Golander, A., and R.E. Fellows, 1977: Studies on ovine placental lactogen secretion by homologous radioimmunoassay. *J. Endocrinol.* 72, 27-34.

Hansel, W., and K. McEntee, 1977: Female reproductive processes. In *Duke's Physiology of Domestic Animals*. (M. Swenson, ed.) U.S.A.: Vail-Ballou Press, Inc.

Harvey, E., 1959: Implantation, development of the foetus and foetal membranes. In *Reproduction in Domestic Animals*. (H. Cole and P. Cupps, ed.) U.S.A.: Academic Press.

Hesseldahl, H., 1971: Ultrastructure of early cleavage stages and preimplantation in the rabbit. *Z. Anat. Entwickl.-Gesch.* 135, 139-155.

Hoffman, L.H.; Davies, J., and V.D. Long, 1975: Hormone-induced crystals and intramitochondrial lamellae in uterine epithelium. In *Electron Microscopic Concepts of Secretion*. (M. Hess, ed.) U.S.A.: John Wiley and Sons.

Hopwood, D., 1970: The reactions between formaldehyde, glutaraldehyde and osmium tetroxide, and their fixation effects on bovine serum albumin and on tissue blocks. *Histochemie* 24, 50-64.

Hull, B.E., and L.A. Staehelin, 1979: the terminal web. A re-evaluation of its structure and function. *J. Cell Biol.* 81, 67-82.

Hurley, T.W., Grisson, F.E., Handwerger, S., and R.E. Fellows, 1977a: Purification and partial characterization of the cyanogen bromide fragments of ovine placental lactogen. *Biochemistry* 16, 5605-5609.

Hurley, T.W., Handwerger, S., and R.E. Fellows, (1977b): Isolation and characterization of ovine placental lactogen. *Biochemistry* 16, 5598-5604.

- Hurst, P.R., Jeffries, K., Eckstein, P., and A.G. Wheeler, 1978: An ultrastructural study of preimplantation uterine embryos of the rhesus monkey. *J. Anat.* 126, 209-220.
- Hutson, J.C., Childs, G.V., and P.J. Gardner, 1979: Considerations for establishing the validity of immunocytochemical studies. *J. Histochem. Cytochem.* 27, 1201-1202.
- Jenkinson, J.W., 1906: Notes on the histology and physiology of the placenta in ungulata. *Proc. Zool. Soc., Lond.* 1, 73-96.
- Jeon, K.W., and J.R. Kennedy, 1973: The primordial germ cells in early mouse embryos: light and electron microscopic studies. *Devel. Biol.* 31, 275-284.
- Josimovich, J.B., and J.A. MacLaren, 1962: Presence in the human placenta and term serum of a highly lactogenic substance immunologically related to pituitary growth hormone. *Endocrinology* 71, 209-220.
- Kaplan, S.L., and M.M. Grumbach, 1964: Studies of a human and simian placental hormone with growth hormone-like and prolactin-like activities. *J. Clin. Endocrinol. Metab.* 24, 80-100.
- Kelly, P.A., Shiu, R.P.C., Friesen, H.G., and H.A. Robertson, 1973: Placental lactogen levels in several species throughout pregnancy. *Endocrinology* 92, Suppl. A-233 (Abstr.).
- Kelly, P.A., Robertson, H.A., and H.G. Friesen, 1974: Temporal pattern of placental lactogen and progesterone secretion in sheep. *Nature* 248, 435-437.
- Kelly, P.A., Tsushima, T., Shiu, R.P.C., and H.G. Friesen, 1976: Lactogenic and growth hormone-like activities in pregnancy determined by radioreceptor assays. *Endocrinology* 99, 765-774.
- King, G.J., Atkinson, B.A., and H.A. Robertson, 1979: Development of the bovine placentome during the second month of gestation. *J. Reprod. Fert.* 55, 173-180.
- Kohmoto, K., and H.A. Bern, 1970: Demonstration of mammatropic activity of the mouse placenta in organ culture and by transplantation. *J. Endocrinol.* 48, 99-107.
- Lawn, A.M., Chiquoine, A.D., and E.C. Amoroso, 1969: The development of the placenta in the sheep and goat: an electron microscope study. *J. Anat.* 105, 557-578.
- Lewis, P.R., and D.P. Knight, 1977: Practical Methods in Electron Microscopy. Staining Methods for Sectioned Material. The Netherlands: North Holland Publishing Co.

Lillie, R.D., 1965: Histopathologic Technic and Practical Histochemistry. U.S.A.; McGraw-Hill Inc.

Lo, C.W., and N.B. Gilula, 1979: Gap junctional communication in the preimplantation mouse embryo. *Cell* 18, 399-409.

Mabuchi, I., and M. Okuno, 1977: The effect of myosin antibody on the division of starfish blastomeres. *J. Cell Biol.* 74, 251-263.

Martal, J., and J. Djiane, 1975: Purification of a lactogenic hormone in sheep placenta. *Biochem. Biophys. Res. Commun.* 65, 770-778.

Martal, J., and J. Djiane, 1977a: The production of chorionic somatomammotrophin in sheep. *J. Reprod. Fert.* 49, 285-289.

Martal, J., and J. Djiane, 1977b: Mammatrophic and growth promoting activities of a placental hormone in sheep. *J. Steroid Biochem.* 8, 415-417.

Martal, J., Djiane, J., and M. Dubois, 1977: Immunofluorescent localization of ovine placental lactogen. *Cell Tiss. Res.* 184, 427-433.

McLaren, A., 1972: The embryo. *In* Embryonic and Fetal Development. (C.R. Austin and R.V. Short, ed.) Great Britain: Cambridge University Press.

Moriarty, G.C., 1975: Electron microscopic-immunocytochemical studies of rat pituitary gonadotrophs: a sex difference in morphology and cytochemistry of LH cells. *Endocrinology* 97, 1215-1225.

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

Nakao, K., Meyer, C.J., and Y. Noda, 1971: Progesterone-specific protein crystals in the endometrium: an electron microscopic study. *Am. J. Obstet. Gynec.* 111, 1034-1038.

Nishino, Y., and Y. Watanabe, 1977: Evidence suggesting the presence of common antigenic determinant between dynein and tubulin. *Biochim. Biophys. Acta* 493, 104-114.

Norberg, H.S., 1973: Ultrastructural aspects of the preattached pig embryo: cleavage and early blastocyst stages. *Z. Anat. Entwickl.* 143, 95-114.

Nowel, M.S., and G.B. Chapman, 1976: The ultrastructure of implanted trophoblast cells of the yellow agouti mouse. *J. Anat.* 122, 177-188.

Olden, K., and K.M. Yamada, 1977: Direct detection of antigens in sodium dodecyl sulfate-polyacrylamide gels. *Analyt. Biochem.* 78, 483-490.

Osada, H., 1976: Human placental lactogen. Further purification and immunohistological localization of human placental lactogen in syncytiotrophoblasts. *Folia endocrinol. jap.* 52, 36-53.

Panigel, M., Kraemer, D.C., Kalter, S.S., Smith, G.C., and R.L. Heberling, 1975: Ultrastructure of cleavage stages and pre-implantation embryos of the baboon. *Anat. Embryol.* 147, 45-62.

Parkening, T.A., 1976: An ultrastructural study of implantation in the golden hamster. I. Loss of the zona pellucida and initial attachment to the uterine epithelium. *J. Anat.* 121, 161-184.

Pearse, A.G.E., 1972: *Histochemistry, Theoretical and Applied*. Great Britain: Churchill Livingstone.

Petrusz, P., Sar, M., Ordronneau, P., and P. DiMeo, 1976: Specificity in immunocytochemical staining. *J. Histochem. Cytochem.* 24, 1110-1112.

Petrusz, P., Sar, M., Ordronneau, P., and P. DiMeo, 1977: Reply to the letter of Swaab *et al*: Can specificity ever be proved in immunocytochemical staining?. *J. Histochem. Cytochem.* 25, 390-391.

Ray, E.E., Averill, S.C., Lyons, W.R., and R.E. Johnson, 1955: Rat placental hormonal activities corresponding to those of pituitary mamotropin. *Endocrinology* 56, 359-373.

Reddy, S., and W.B. Watkins, 1978a: Purification and some properties of ovine placental lactogen. *J. Endocr.* 78, 59-69.

Reddy, S., and W.B. Watkins, 1978b: Immunofluorescence localization of ovine placental lactogen. *J. Reprod. Fert.* 52, 173-174.

Reynolds, E.S., 1963: The use of lead citrate at high pH as an electron opaque stain in electron microscopy. *J. Cell Biol.* 17, 207-212.

Rowson, L.E.A., and R.M. Moor, 1966: Development of the sheep conceptus during the first fourteen days. *J. Anat.* 100, 777-785.

Rowson, L.E.A., and R.M. Moor, 1967: The influence of embryonic tissue homogenate infused into the uterus on the lifespan of the corpus luteum in the sheep. *J. Reprod. Fert.* 13, 511-516.

Schlafke, S., and A.C. Enders, 1963: Observations on the fine structure of the rat blastocyst. *J. Anat. Lond.* 97, 353-360.

Schlafke, S., and A.C. Enders, 1967: Cytological changes during cleavage and blastocyst formation in the rat. *J. Anat.* 102, 13-32.

Schliwa, M., Osborn, M., and K. Weber, 1978: Microtubule system of isolated fish melanophores as revealed by immunofluorescence microscopy. *J. Cell Biol.* 76, 229-236.

Sheridan, M.N., and S.H. Walker, 1975: Fine-structural features of pineal gland development. *In* *Electron Microscopic Concepts of Secretion*. (M. Hess, ed.) U.S.A.: John Wiley and Sons.

Sherwood, L.M., Handwerger, S., McLaurin, W.D., and M. Lanner, 1971: Amino acid sequence of human placental lactogen. *Nature New Biology* 233, 59-61.

Smith, M.S.R., and I.B. Wilson, 1971: Histochemical observations on early implantation in the mouse. *J. Embryol. exp. Morph.* 25, 165-174.

Spurr, A.R., 1969: A low-viscosity epoxy resin embedding medium for electron microscopy. *J. Ultrastruct. Res.* 26, 31-43.

Staehelin, L.A., and B.E. Hull, 1978: Junctions between living cells. *Sci. Am.* 238, 140-152.

Swaab, D.F., and C.W. Pool, 1975: Specificity of oxytocin and vasopressin immunofluorescence. *J. Endocr.* 66, 263-272.

Swaab, D.F., Pool, C.W., and F.W. Van Leeuwen, 1977: Can specificity ever be proved in immunocytochemical staining? *J. Histochem. Cytochem.* 25, 388-390.

Talamantès, F. Jr., 1975: Comparative study of the occurrence of placental prolactin among mammals. *Gen. Comp. Endocrinol.* 27, 115-121.

Turner, C.D., and J.T. Bagnara, 1971: *General Endocrinology*. U.S.A.: W.B. Saunders Co.

Van Blerkom, J., Manes, C., and J.C. Daniel, Jr., 1973: Development of preimplantation embryos *in vivo* and *in vitro*. *Devel. Biol.* 35, 262-282.

Watkins, W.B., and S. Reddy, 1980: Ovine placental lactogen in the cotyledonary and intercotyledonary placenta of the ewe. *J. Reprod. Fert.* 58, 411-414.

Wimsatt, W.A., 1951: Observations on the morphogenesis, cytochemistry and significance of the binucleate giant cells of the placenta of ruminants. *Amer. J. Anat.* 89, 233-282.

Wintenberger-Torrès, S., and J.-E. Fléchon, 1974: Ultrastructural evolution of the trophoblast cells of the pre-implantation sheep blastocyst from day 8 to day 13. J. Anat. 118, 143-153.

Wu, J.-T., and R.K. Meyer, 1974: Ultrastructural changes of rat blastocysts induced by estrogen during delayed implantation. Anat. Rec. 179, 253-272.

Zimmerman, E.A., Hsu, K.C., Ferin, M., and G.P. Kozlowski, 1974: Localization of gonadotropin-releasing hormone (Gn-RH) in the hypothalamus of the mouse by immunoperoxidase technique. Endocrinology 95, 1-8.

END

0	7	1	0	9	1	8	2
---	---	---	---	---	---	---	---

FIN