

https://dspace.org/

Analysis of vascular morphogenesis in the early chick embryo

Kessel, Joan

2015-05-13
http://hdl.handle.net/10539/17736

Downloaded from a DSpace repository. https://dspace.org/

ANALYSIS OF VASCULAR MORPHOGENESIS IN [illegible]

Joan Kessel

A thesis submitted to the Faculty of Science,
University of the Witwatersrand, Johannesburg,
in fulfilment of the requirements for the
degree of Doctor of Philosophy

Johannesburg
August, 1981

DECLARATION BY CANDIDATE

I hereby declare that this thesis is my own work and that it has not been submitted to any other university.

ABSTRACT

The present study investigates the cellular events involved in the organization of a mass of mesenchymal cells into a three-dimensional pattern of blood vessels, blood cells, and coelomic linings. This study has been carried out in both the AP and AOV, affording an integrated understanding of this morphogenesis throughout the blastoderm. Attention has been focused on the dorso-ventral and proximo-distal polarities in the developmental properties of the components of this system, and the roles of the associated germinal layers (the ectoderm and endoderm) in these two regions in the temporally and spatially appropriate creation of this pattern.

The histological observations of the morphogenesis of this system (Chapter Two) are essentially in agreement with the currently accepted descriptions in the literature (e.g., Edmonds, 1966; Gonzalez-Crussi, 1971; Murphy and Carlson, 1978), but furthermore, explain the discrepancies which have stemmed from some of the older literature (Sabin, 1917, 1920; Sugiyama, 1926; Houser *et al*, 1961). In analysing the effects of the component germinal layers and other microenvironmental influences in moulding the morphogenesis of this system, the following types of experiments have been performed:

1. Radioactively labelled mesodermal fragments or blood-islands have been autotopically or heterotopically transplanted into the AP or AOV of non-radioactive host blastoderms of known ages. After an overnight culturing period, the developmental fates of the transplanted cells have been followed by autoradiography of histological serial sections (Chapters Three and Seven).
2. Small fragments of the blastoderm, composed of given combinations of the germinal layers from the AP and/or AOV of blastoderms of known ages have been cultured for 72 hours in a small drop of culture medium on the surface of coverslips below cavity slides. Thereafter, the extent of erythropoiesis of the explants has been determined by means of a haemoglobin staining procedure (the o-dianisidine stain). Investigations have been made of:

(a) the influence of the ectoderm and endoderm from the AP and AOV of blastoderms of various ages (stage 1-2 to stage 8-9) on mesodermal erythropoiesis (Chapter Four);

(b) the effect of the enzymatic removal of the extracellular matrix components, hyaluronate and collagen, on mesodermal erythropoiesis (Chapter Five).

(c) the effect of physically flattening the explants on mesodermal erythropoiesis (Chapter Six).

In developmental harmony with the dorso-ventral polarity in the morphogenesis of this system, it is found not only that the opaca endoderm exerts a stimulatory effect on mesodermal erythropoiesis (see also Wilt, 1965a and Miura and Wilt, 1969), but that the opaca ectoderm exerts an inhibitory effect on mesodermal erythropoiesis (Chapter Four). This inhibitory effect of the opaca ectoderm is coupled with the tendency of the opaca ectoderm-plus-mesoderm explants, in the absence of the opaca endoderm, to spread out and become flattened in culture. This finding is in line with the histological observation (Chapter Two) that the opaca ectoderm is a surface under which the presumptive coelomic lining cells spread out and form a flattened sheet, and is not a surface against which the blood-island cells clump together. The finding that the opaca endoderm counteracts this spreading tendency and causes a significant enhancement in the erythropoietic differentiation of the explants (Chapter Four), is in line with the histological observation (Chapter Two) that the opaca endoderm is a surface against which the blood-island cells normally clump together.

In developmental harmony with the proximo-distal gradation in the morphogenesis of this system, it is found that whereas the opaca environment is overall favourable for mesodermal erythropoiesis, the pellucida environment is unfavourable for mesodermal erythropoiesis. Indeed, the pellucida ectoderm is likewise found to exert an inhibitory effect on mesodermal erythropoiesis which the pellucida endoderm, unlike the opaca endoderm, is unable to counteract (Chapter Four). The inhibitory effect of the pellucida environment on mesodermal erythropoiesis is in line with the observation that the blood-islands (angioblasts) which form in the AP tend to differentiate into empty blood vessels only, and do not give rise to blood cells (Chapter Two).

In a preliminary investigation of the effect on mesodermal erythropoiesis of the enzymatic removal of the extracellular matrix components hyaluronate and collagen (Chapter Five), it is found that whereas the removal of hyaluronate has no apparent effect on mesodermal erythropoiesis, the removal of collagen appears to counteract the stimulatory influence of the space endoderm on mesodermal erythropoiesis.

Dedicated to my dear parents,
Mavis and Benny Kessel,
with gratitude, respect and love.

"We were curious. Our curiosity was not limited . . . we wanted to see everything our eyes would accommodate, to think what we could, and, out of our seeing and thinking, to build some type of structure in modeled imitation of the observed reality."

John Steinbeck,
"The Log from the Sea of Cortez"

ACKNOWLEDGEMENTS

I wish to acknowledge my supervisor, Professor Barry C. Fabian, a true and dedicated scientist and mentor, for sharing with me his enquiring mind and fascination for research, for his constant interest and enthus asm in my work, and for his wealth of insight and imaginatio in dealing with the various aspects of the investigation. I wish also to acknowledge both Professor Fabian and Professor B.I. Balinsky, emeritus Professor and former Head of this department, from whom I acquired my solid foundation in, and deep love for, the subject of Embryology. Furthermore, I wish to thank both Professor Fabian and Professor Hugh E. Paterson, Head of this department, for the excellent laboratory facilities made available to me in the B.I. Balinsky Laboratory of Developmental Biology.

I sincerely thank the following people for their interest in my work, and from whom I learned some important experimental techniques:

Professor Ann Andrew. Professor of Embryology, Anatomy Department, Medical School of this university;

Dr. Beverley Kramer, Senior Lecturer, Department of General Anatomy of this university (formerly of e Anatomy Department);

Professor Hefzibah Eyal-Giladi, Head of the Zoology Department, Hebrew University of Jerusalem, Israel; and

Dr. Alan Thornley, Senior Lecturer of this department.

I wish also to thank:

Dr. M.J. Witcomb and staff of the Electron Microscope Unit of this university, and Dr. J.T. Fourie and staff of the Electron Microscope Division. National Physical Research Laboratory, C.S.I.R., for the use of their excellent services;

Mr. Michael Howard-Tripp and staff of the Central Animal Unit of this university for so efficiently providing me with the eggs I required;

Ms Walli Maier and Mr. J.V. Thompson, the present and previous Photographers of this department, for so painstakingly processing and printing all my photographs;

Mr. Enzo Vaccaro, Chief Technician, and Mr. Moses Banda, Technician of this department, for so efficiently providing me with all the chemicals and apparatus I required; and

Mrs. E.F. Patterson, Secretary of this department, for so carefully typing this thesis.

I wish to acknowledge the University of the Witwatersrand and the C.S.I.R. for the bursaries I received, and the Freda Lawenski Trust for the scholarship I received.

My colleagues and very good friends, Arielle Mills and Sue Kidson, are thanked for their relevant comments during our numerous discussions, and for helping to create in the B.I. Balinsky Laboratory the warm, convivial atmosphere so conducive to good ork.

Finally, I wish to express my deepest gratitude to my parents, Mavis and Benny Kessel, to whom this thesis is dedicated.

TABLE OF CONTENTS

	page
Declaration by Candidate	ii
Abstract	iii
Dedication	vi
Quotation	vii
Acknowledgements	viii
Table of Contents	x
List of Tables	xviii

CHAPTER ONE

GENERAL INTRODUCTION

1.1	Introduction and General Aim	1
1.2	The Yolk Sac Vascular System of the Chick Blastoderm	2
1.2.1	The functions of the yolk sac vascular system	2
1.2.2	The morphogenesis of the yolk sac vascular system: The current view	3
1.2.3	Previous disparities in the literature with respect to the descriptions of blood-island differentiation	10
1.2.4	The formation of the extraembryonic coelom	13
1.3	Tissue Interactions during Yolk Sac Vasculogenesis	14
1.3.1	Experimental studies of the influences exerted by the endoderm and mesoderm on each other during the course of differentiation	14
1.4	The Role of the Extracellular Matrix during Embryogenesis	17
1.5	General Overview of the Present Investigation	18

CHAPTER TWO

page

THE MORPHOGENESIS OF THE CHICK YOLK SAC VASCULAR SYSTEM AND EXTRAEMBRYONIC COELOM AS STUDIED WITH THE LIGHT- AND SCANNING ELECTRON MICROSCOPES

2.1 Introduction 19

2.2 Materials and Methods 20

2.2.1 Eggs and embryos 20

2.2.2 Preparation of tissue for scanning electron microscopy 20

2.2.3 Preparation of tissue for light microscopy 21

1 Preparation of wax embedded serial cross sections 21

2 Preparation of plastic embedded sections 22

3 Preparation of whole-mounted blastoderms 22

2.3 Observations 22

2.3.1 The origin and migration of the primary mesenchymal cells 22

2.3.2 The cellular events involved in vasculogenesis and coelomogenesis in the area opaca vasculosa 30

1 The transformation of some of the mesodermal cells into the yolk sac blood-islands 31

2 The organization of the rest of the mesenchymal cells into the linings of the extraembryonic coelom 34

3 The transformation of the peripheral cells of the blood-islands into the endothelial linings 47

4 The joining up of the blood-islands into a plexus 48

5 Further stages in the morphogenesis of the blood vessels and intravascular blood-island cells 49

		page
	The dissolution of the intra-vascular blood-island cells	54
	Further observations on the yolk sac blood vessels	62
	Further observations on the linings of the extraembryonic coelom	77
	General	79
3	The cellular events involved in vasculogenesis and coelomogenesis in the area pellucida	80
	The morphogenesis of the extra-embryonic blood vessels in the area pellucida	80
	The morphogenesis of the extra-embryonic coelom in the area pellucida	87
	The development of the first embryonic erythrocytes of the chick blastoderm	91
	Background	91
	Scanning electron microscopic observations of 3-day and 5-day blood cells	94
	Observations of the primordial germ cells	101
.+	Discussion	109
	The origin of the primary mesenchymal cells	109
.4.	The centrifugal migration of the primary mesenchymal cells	111
2.4.3	Pattern formation during the morphogenesis of the yolk sac vascular system	120
	1 Models explaining the patterns of morphogenesis	120
	2 Gradients and/or polarities in the pattern of morphogenetic events	121
	3 Possible explanations for previous discrepancies in the descriptions of blood-island differentiation	127

page

2.4.4 The determination and erythropoietic differentiation of the blood-island cells .....

2.4.5 Discussion of the origin and developmental fates of the yo'k sac-derived erythropoietic cells and their relationships to the intraembryonic haemopoietic cells .....

CHAPTER THREE

AN INVESTIGATION OF THE EFFECTS OF THE PELLUCIDA AND OPA[illegible] ENVIRONMENTS ON THE PATHWAYS OF DIFFERENTIATION OF THE MESODERMAL CELLS, BY MEANS OF THE TRANSPLANTATION OF MESODERMAL FRAGMENTS FROM ONE R.GION OF THE BLASTODE[illegible]M INTO ANOTHER

3.1 Introduction .....

3.2 Materials and Methods .....

3.2.1 Sterile techniques .....

3.2.2 Tissue transplantation experiments .....

1 Explantation of the host blastoderms onto solid organ culture medium .....

2 P[illegible]paration of the solid organ culture medium .....

3 Preparation of radioactively labelled donor tissue for transplantation .....

4 Culturing of the host blastoderms .....

5 Processing of the host blastoderms for autoradiography .....

3.3 Experiments and Results .....

3.3.1 Experiments to investigate the effects of the pellucida and opaca environments on the pathways of differentiation of the mesodermal cells .....

3.3.2 Control experiments .....

3.4 Discussion 160

page

3.4.1 The pathways of differentiation of the heterotopically transplanted mesodermal cells 160

3.4.2 Discussion of the control experiments 163

3.4.3 Discussion of the experimental techniques 164

CHAPTER FOUR

AN [illegible] INVESTIGATION OF THE EFFECTS OF THE COMPONENT CELL TYPES OF THE AREA PELLUCIDA AND AREA OPACA VASCULOSA ON THE ERYTHROPOIETIC DIFFERENTIATION OF THE MESODERMAL CELLS, BY MEANS OF THE COVERSLIP CULTURE OF BLASTODERM FRAGMENTS IN SMALL DROPS OF MEDIUM

4.1 Introduction 166

4.2 Materials and Methods 168

4.2.1 The coverslip culture of blastoderm fragments in small drops of culture medium 168

1 Preparation of blastoderm fragments 169

2 Preparation of the liquid culture medium 169

3 Method of coverslip culture 170

4 Fixation, staining and processing of the coverslip cultures 170

4.3 Experiments and Results 172

4.3.1 An investigation of the ability of the stage 8-9 pellucida mesodermal fragments to give rise to blood cells when cultured alone or in combination with the other germinal layers from the AP and AOV 172

1 Culture experiments 173

2 Summary of the investigation of the erythropoietic potential of the stage 8-9 pellucida mesodermal tissue 185

4.3.2 An investigation of the effects of the stage 4 opaca endoderm and ectoderm on the erythropoietic differentiation of the stage 4 opaca mesoderm 187

page

1 Culture experiments 189

2 Summary of the investigation of the effects of the stage 4 opaca endoderm and ectoderm on the erythropoietic differentiation of the stage 4 opaca mesoderm 195

4.3.3 An investigation of the erythropoietic potential of the stage 4 pellucida mesoderm when cultured alone or in combination with the other germinal layers from the AP and AOV 198

1 Culture experiments 199

2 Summary of the investigation of the erythropoietic potential of the stage 4 pellucida mesoderm 208

4.4 An investigation of the effects of the stage 1-2 opaca ectoderm and endoderm on mesodermal erythropoiesis 209

1 Culture experiments 210

2 Summary of the investigation of the effects of stage 1-2 opaca ectoderm and endoderm on mesodermal erythropoiesis 221

4.4 Discussion 222

4.4.1 The effects of the stage 4 opaca endoderm and ectoderm on mesodermal erythropoiesis 223

4.4.2 The effects of the stage 4 pellucida endoderm and ectoderm on mesodermal erythropoiesis 228

4.4.3 The pluripotency of the stage 8-9 pellucida mesodermal cells 232

4.4.4 The effect of the stage 1-2 opaca endoderm and ectoderm on mesodermal erythropoiesis 239

page

CHAPTER FIVE

THE ROLE OF THE COMPONENTS OF THE EXTRACELLULAR MATRIX, HYALURONATE AND COLLAGEN, IN YOLK SAC VASCULOGENESIS AS INVESTIGATED BY THE COVERSLIP CULTURE OF BLASTODERM FRAGMENTS IN SMALL DROPS OF MEDIUM

5.1 Introduction 242

5.2 Materials and Methods 243

5.3 Experiments and Results 244

5.3.1 An investigation of the effects of the removal of hyaluronate (by hyaluronidase treatment) on mesodermal erythropoiesis 244

1 Culture experiments 246

5.3.2 An investigation of the effects of the removal of collagen (by collagenase treatment) on mesodermal erythropoiesis 256

1 Culture experiments 259

5.4 Discussion 268

5.4.1 Discussion of the investigation of the effect of the removal of hyaluronate (by hyaluronidase treatment) on mesodermal erythropoiesis 268

5.4.2 Discussion of the investigation of the effect of the removal of collagen (by collagenase treatment) on mesodermal erythropoiesis 269

CHAPTER SIX

AN INVESTIGATION OF THE EFFECT OF FLATTENING EXPLANTS BY PHYSICAL MEANS ON MESODERMAL ERYTHROPOIESIS: COVERSLIP CULTURE OF BLASTODERM FRAGMENTS IN SMALL DROPS OF MEDIUM

6.1 Introduction 273

6.2 Materials and Methods 274

6.3 Experiments and Results 274

6.3.1 Culture experiments 275

6.4 Discussion 286

page

CHAPTER SEVEN

ARE THE BLOOD-ISLAND CELLS IRREVERSIBLY RESTRICTED TO THE FORMATION OF THEIR NORMAL PROGENY LINEAGES (ENDOTHELIUM AND BLOOD CELLS) OR ARE THEY STILL ABLE TO CHANGE THEIR PATHWAYS OF DIFFERENTIATION? AN INVESTIGATION BY THE TRANSPLANTATION OF BLOOD-ISLANDS INTO HOST BLASTODERMS

7.1 Introduction 292

7.2 Materials and Methods 29

7.2.1 Preparation of radioactively labelled blood-islands 295

7.2.2 Transplantation of radioactively labelled blood-island fragments into unlabelled host blastoderms 297

7.3 Experiments and Results 297

7.4 Discussion 301

CHAPTER EIGHT

CONCLUDING DISCUSSION

8.1 Overview and Some Hypotheses 302

8.1.1 The morphogenetic patterning of the yolk sac vascular system 304

8.2 Conclusion 319

REFERENCES 321

page

LIST OF TABLES

4.1 Table summarizing the results of the study to investigate the ability of the stage 8-9 pellucida mesodermal fragments to give rise to blood cells when cultured alone or in combination with the other germinal layers from the AP and AOV 186

4.2 Table summarizing the results of the study to investigate the effects of the stage 4 opaca endoderm and ectoderm on the erythropoietic differentiation of the stage 4 opaca mesoderm 197

4.3 Table summarizing the results of the study to investigate the erythropoietic potential of the stage 4 pellucida mesoderm when cultured alone or in combination with the other germinal layers from the AP and AOV 207

4.4 Table summarizing the results of the study to investigate the effects of the stage 1-2 opaca endoderm and ectoderm on mesodermal erythropoi 220

5.1 Table summarizing the results of the study to investigate the effects of the removal of hyaluronate (by 0,2 hyaluronidase treatment) on mesodermal erythropoiesis 255

5.2 Table summarizing the results of the study to investigate the effects of the removal of collagen (by 0,2% collagenase treatment) on mesodermal erythropoiesis 267

6.1 Table summarizing the results of the study to investigate the effect of flattening the explants by physical means on mesodermal erythropoiesis 285

CHAPTER ONE

GENERAL INTRODUCTION

1.1 Introduction and General Aim

At the cellular level the creation of form during embryonic development is seen to take place by means of changes in the shapes of cells, morphogenetic movements, cell proliferation, and involves the organization of interacting cells into predetermined patterns in time and space. Temporally coupled with this elaboration of tissue architecture is the acquisition by the individual cells of their own characteristic functional specializations.

The early development of the yolk sac vascular system of the chick blastoderm involves the transformation of an irregular mass of migrating mesodermal cells into an organized system consisting of anastomosing tubes containing free cells in large cell-lined spaces (see Fig. 1.1). The anastomosing tubes are the yolk sac blood vessels formed from flattened endothelial cells. The free cells within the vessels are the first developing blood cells. The large cell-lined spaces constitute the extraembryonic coelom, lined with sheets of flattened coelom-lining cells (Fig. 1.1).

The organization of these morphologically identical mesodermal cells into this diversity of cellular specialization occurs within a relatively short period of time, and thus provides a very useful model system with which to study the genesis of form and function.

The aim of the present study is to describe the morphogenesis and analyse some of the causal events which result in the formation of this differentiated cellular pattern of single cells, cellular tubes, and sheets of cells. Specifically, it is of interest to investigate the extent to which the progenitor mesodermal cells are predetermined to form the different parts of the pattern, and the extent to which the environment into which they migrate influences the course of differentiation which they will follow.



(From Balinsky, 1981, after Wieman).

Figure 1.1

The chick yolk sac vascular system comprises a system of tubes (blood vessels, bv), free cells (intravascular blood cells, bl), and large cell-lined spaces (the extraembryonic coelom, c). The blood vessels (bv) are in juxtaposition to the endoderm (en), and are separated from the ectoderm (ec) by the coelom (c)

bl, blood cells; bv, blood vessel; c, coelom; e, vessel endothelium; ec, ectoderm; en, endoderm; som, dorsal lining of coelom; spm, ventral lining of coelom.

1.2 The Yolk Sac Vascular System of the Chick Blastoderm

1.2.1 The Functions of the yolk sac vascular system

The yolk sac vascular system is an extraembryonic 'organ' which plays a vital role in several important aspects of embryonic development. Firstly, the formation of the yolk sac vascular system precedes the formation of the embryo proper, allowing it to play an important role in the development of the embryo itself since it serves to mobilize nutrients from the yolk sac to the developing embryo (see Patten, 1971; Romanoff, 1960). In addition, the yolk sac

vascular system is the first haemopoietic organ to develop (see Metcalf and Moore, 1971; Dieterlen-Lièvre, 1978) and it therefore supplies the embryo with its first embryonic blood cells. Furthermore, it has recently been shown that the yolk sac subsequently becomes recolonized by embryo-derived haemopoietic stem cells, and therefore serves also as a secondary site for the maturation of further populations of blood cells (see Dieterlen-Lièvre, 1978; Martin *et al*, 1978; Beaupain *et al*, 1979).

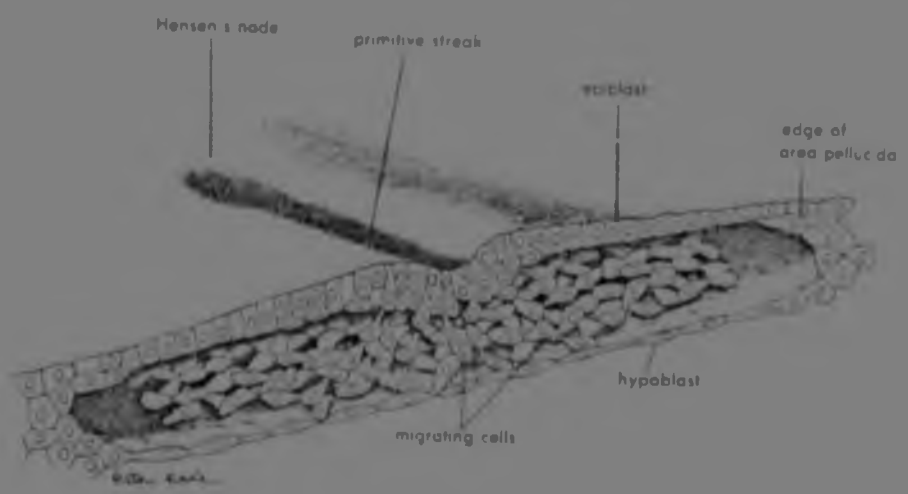
The yolk sac is also the site of origin of the primordial germ cells from the yolk sac endoderm (Swift, 1914). The blood vessels of the yolk sac play an important role in the passage of these primordial cells into the body of the embryo. The primordial germ cells then migrate into the developing gonads where they subsequently give rise to the gametes of the new individual (Swift, 1914; Meyer, 1964; Clawson and Domm, 1967; Fujimoto *et al*, 1976a,b).

1.2.2 The morphogenesis of the yolk sac vascular system: The current view

The following description of the morphogenesis of the yolk sac vascular system is based on the currently accepted views on the morphogenesis of this system, as described in the studies of Murray (1932), Mato *et al* (1964), Edmonds (1966), Gonzalez-Crussi (1971), and Murphy and Carlson (1978), and as outlined in the textbooks of Patten and Carlson (1974) (see also Patten, 1971), and Balinsky (1981). Although disparities have existed in the literature from some of the earlier descriptions of yolk sac vasculogenesis (Sabin, 1917, 1920; Sugiyama, 1926; Houser *et al*, 1961), these have subsequently been resolved in the more recent literature (Edmonds, 1966; Gonzalez-Crussi, 1971; Murphy and Carlson, 1978). A review of these disparities is presented in Section 1.2.3 below. The aim of the present section is to provide the reader with a clear overall picture of the currently accepted views on the morphogenesis of this system.

The yolk sac vascular system is formed from mesodermal cells which originate in the epiblast of the early chick blastoderm (see Rosenquist, 1966). With the formation of the primitive streak, these cells become translocated into the space between the epiblast and

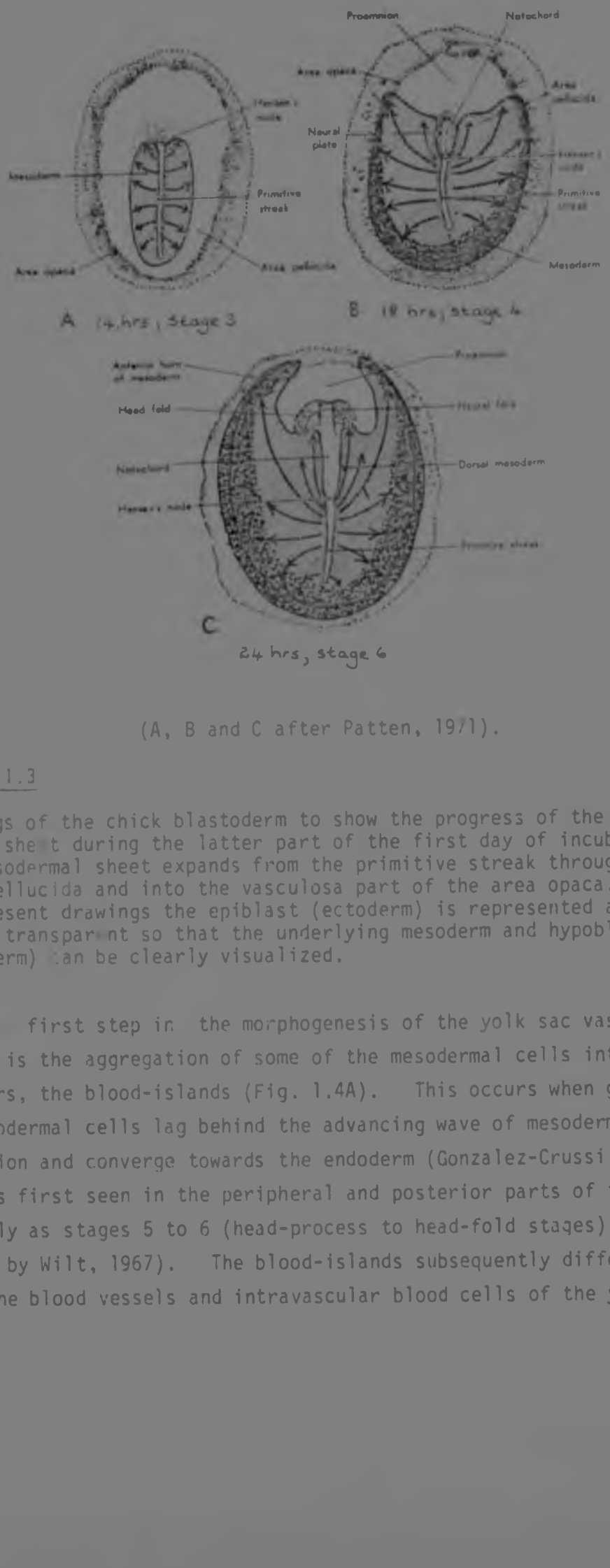
hypoblast (Fig. 1.2), and they form an irregular sheet of cells which spreads centrifugally through the area pellucida (AP) into the yolk sac itself, the area opaca (Figs. 1.2, 1.3) (see Patten and Carlson, 1974; Balinsky, 1981). The boundary between the proximal (vasculosa) and the distal (vitellina) parts of the area opaca is established by the extent of the migration of this mesodermal sheet into the area opaca (see Patten and Carlson, 1974). The proximal zone into which the mesoderm expands is known as the area opaca vasculosa (AOV), because it is from the mesoderm in this region that the yolk sac vascular system will develop. The distal zone of the area opaca remains free of this expanding mesodermal sheet, and is termed the area opaca vitellina (Fig. 1.3C). In this latter zone the epiblast and hypoblast remain closely apposed to each other and do not become separated by extracellular spaces (see Romanoff, 1960).



(From Balinsky, 1981)

Figure 1.2

Anterior half of the area pellucida of a chick blastoderm cut transversely to show the migration of the mesodermal cells from the primitive streak. These mesodermal cells originate in the epiblast, but become translocated through the primitive streak into the space between the epiblast and hypoblast.

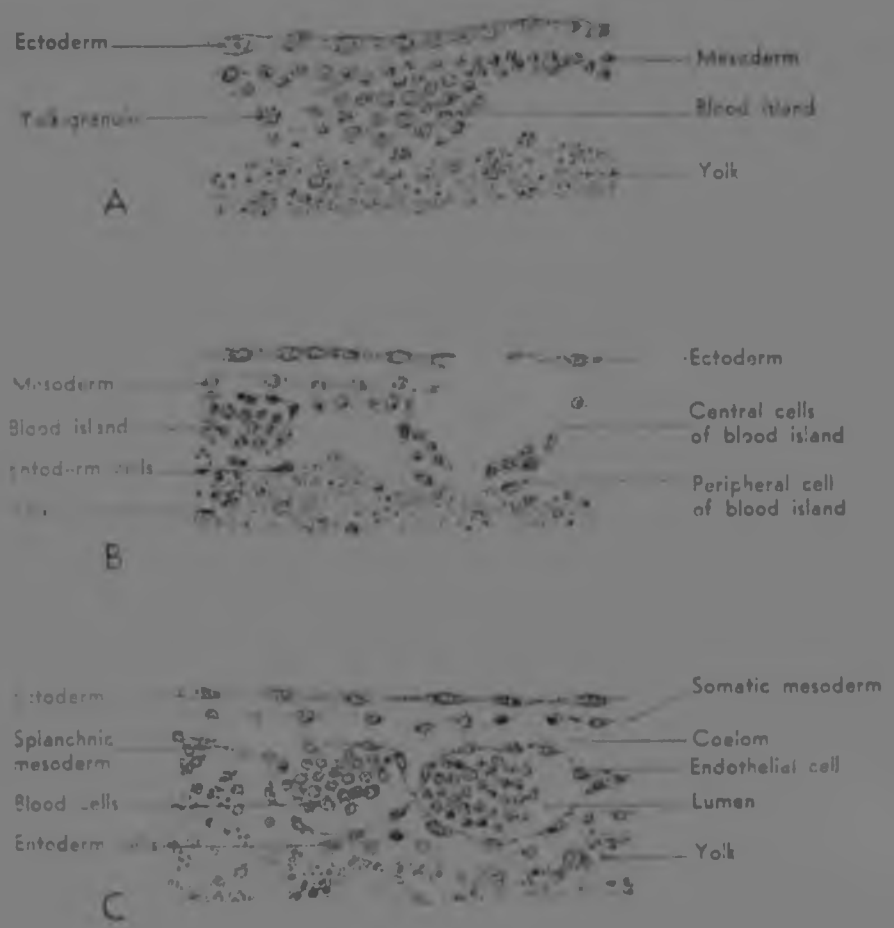


(A, B and C after Patten, 1971).

Figure 1.3

Drawings of the chick blastoderm to show the progress of the mesodermal sheet during the latter part of the first day of incubation. The mesodermal sheet expands from the primitive streak through the area pellucida and into the vasculosa part of the area opaca. In the present drawings the epiblast (ectoderm) is represented as being nearly transparent so that the underlying mesoderm and hypoblast (endoderm) can be clearly visualized.

first step in the morphogenesis of the yolk sac vascular system is the aggregation of some of the mesodermal cells into cell clusters, the blood-islands (Fig. 1.4A). This occurs when groups of mesodermal cells lag behind the advancing wave of mesodermal extension and converge towards the endoderm (Gonzalez-Crussi, 1971). This is first seen in the peripheral and posterior parts of the AOV as early as stages 5 to 6 (head-process to head-fold stages) (see review by Wilt, 1967). The blood-islands subsequently differentiate into the blood vessels and intravascular blood cells of the yolk sac



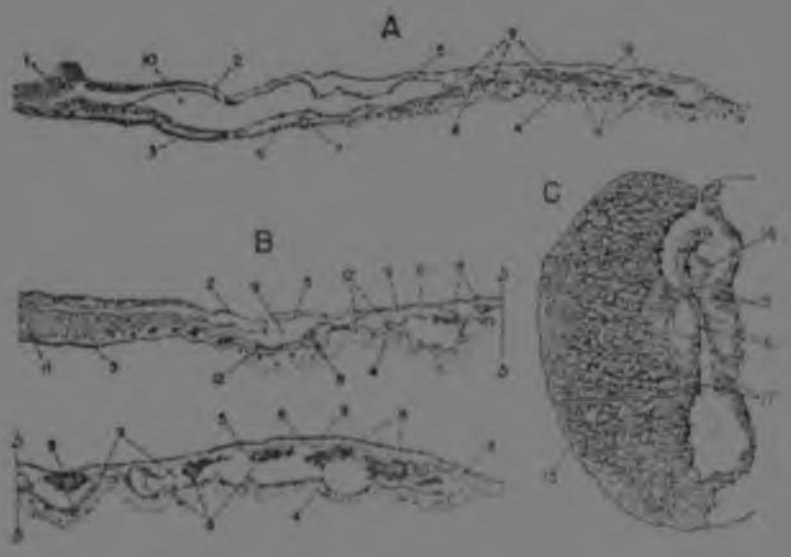
and Carlson, 1974).

Figure 1.5

organization of blood-islands in the area in their differentiation (see text).

stem (Murphy, ; Mat , 1964; Edmond , 1964, Gonzalez-Crussi, ; Small and Davies, 1972). Meanwhile, the rest of the mesodermal cells in the sheet give rise to the linings of the extraembryonic coelom (Sabin, 1920; Gonzalez-Crussi, 1971; Murphy and Carlson, 1974) (Fig. 1.5).

The currently accepted description of this morphogenesis is as follows: "At the time of their first appearance the blood-islands are irregular clusters of mesodermal cells lying in intimate contact with the yolk sac endoderm." (Figs. 1.4A, 1.5A). "When the lateral mesoderm becomes split, forming the somatic and splanchnic layers with the coelom between, the blood-islands lie in the splanchnic mesoderm adjacent



(From Romanoff, 1960, after Rückert and Mollier, and Chapman).

Figure 1.5

Early stages in the differentiation of the yolk sac vascular system of the chick blastoderm.
A, cross section of a stage 8-9 chick blastoderm (6 somite stage), taken at a level anterior to the somites (x 60).
B, cross section of a stage 10-11 chick blastoderm (12 somite stage), taken at the level of the caudal part of the body (x 60).
C, surface view of a stage 12 blastoderm (16 somite stage) to show the embryo and the left half of the vascular system (x 10).

1, neural plate; 2, ectoderm; 3, endoderm of area pellucida; 4, endoderm of area opaca; 5, somatic mesoderm; 6, splanchnic mesoderm; 7, anlage of pellucida blood vessel; 8, blood-island; 9, extraembryonic coelom; 10, pericardial cavity; 11, primitive streak; 12, pellucida blood vessel; 13, terminal sinus; 14, heart; 15, vitelline vein; 16, dorsal aorta; 17, capillary network preceding the formation of the vitelline artery.

to the endoderm. In embryos of 3 to 5 somites (stages 7+ to 8), fluid-filled spaces begin to appear in the blood-islands with the result that in each blood-island the peripheral cells are separated from the central ones." (Fig. 1.4B). "As the fluid accumulates and the spaces expand, the peripheral cells become flattened and pushed outward, but they remain adherent to each other and completely enclose the central cells. At this stage the single layer of peripheral cells may be regarded as constituting the endothelial wall of a primitive blood channel." (Fig. 1.4C). "Extension and anastomosis of neighbouring blood-islands which have undergone similar differentiation results in the establishment of a network of communicating vessels." (Fig. 1.5C).



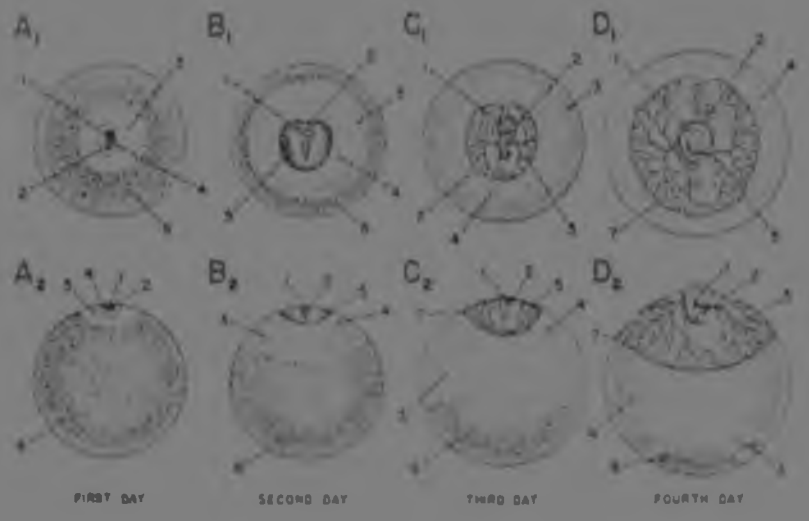
Figure 1.6

Light micrograph of a whole-mounted 2 day chick blastoderm to show the fully functional yolk sac circulation. Blood enters the body of the embryo (E) through the vitelline veins (V) and leaves the body through the vitelline arteries (V). (These main vessels run parallel to each other). At the periphery of the area opaca va.[illegible]losa (AOV) is the terminal sinus (T) which completes the circul[illegible]loop. The terminal sinus (T) also marks the boundary between t[illegible]rea opaca vasculosa (AOV) and the area opaca vitellina (VIT). [illegible]he heart of the embryo is indicated with an arrow.

AOV, area opaca vasculosa; AP, area pellucida; E, embryo; V, vitelline arteries and veins; T, terminal sinus; VIT, area opaca vitellina; arrow, heart.

"Meanwhile, the cells enclosed in the primitive blood channels start to become separated from each other and rounded." (Patten and Carlson, 1974 pages 185-186). These cells are the first developing blood cells of the chick blastoderm (Figs. 1.1, 1.4C, 1.5B).

Although the morphogenesis of the yolk sac vascular system first starts occurring in the AOV, there is a progressive differentiation of the blood-islands *in situ* which gradually extends towards the body of the embryo (Fig. 1.5). Finally the yolk sac blood vessels join up with the vessels of the embryo itself. As the functional differentiation of the heart progresses, the circulation of the yolk sac vascular system gradually



(From Romanoff, 1960, after Duval).

Figure 1.7

Successive stages in the growth of the yolk sac over the surface of the yolk during the first four days of the chick's development.

A1, A2, first day - stage 5; B1, B2, second day - stage 11;
C1, C2, third day - stage 19; D1, D2, fourth day - stage 23.
1, embryo; 2, area pellucida; 3, area vitellina externa;
4, area vitellina interna; 5, area opaca vasculosa; 6, yolk;
7, terminal sinus.

commences, and it becomes functional during the second day of incubation (see Patten and Carlson, 1974; Balinsky, 1981). Haemodynamic forces now become operative, which to a large extent determine the formation of the arteries and veins (Hughes, 1943). By gradual transition the stage of the definitive circulation is reached in which the flow paths to and from the heart are directed through the most direct and efficient of the newly differentiated blood channels (Fig. 1.6).

Successive stages of the morphogenesis of the yolk sac vascular system are shown in Fig. 1.7.

1.2.3 Previous disparities in the literature with respect to the descriptions of blood-island differentiation

As indicated in Section 1.2.2 above, disparities have existed in the literature with respect to the descriptions of the morphological events involved in yolk sac vasculogenesis. These disparities have stemmed from the descriptions of Sabin (1917, 1920), Sugiyama (1926), and Houser *et al* (1961).

These workers carried out extensive observations of vasculogenesis in the living blastoderm using bright field- (Sabin, 1917, 1920; Sugiyama, 1926) or phase contrast- (Houser *et al*, 1961) light microscopy. This is in contrast to the work of all the other authors in the field who used fixed and stained light microscopic- or electron microscopic sections. Sabin and Houser *et al* believed it was more informative to study the morphogenetic events in the living blastoderm as they were occurring rather than to base their observations on fixed and stained histological material.

The main drawback of the approach of Sabin, Sugiyama and Houser is that they were confined to observing the morphological events in a transparent part of the blastoderm. The AOV, being opaque, could not be viewed in the living state. Therefore all the descriptions of Sabin (1917, 1920), Sugiyama (1926) and Houser *et al* (1961) were based on observations in the more transparent AP. Nevertheless, these authors used their observations in the AP as a basis for describing vasculogenesis in the whole of the yolk sac.

According to these authors, the original blood-islands first became transformed into hollow fluid-filled vessels which were initially free of intravascular blood cells (Sabin, 1917, 1920; Sugiyama, 1926; Houser *et al*, 1961). This occurred as a result of the formation of fluid filled cavities in the centre of the blood-islands which enlarged and coalesced with each other, followed by the degeneration of the central cells of the blood-islands into this fluid. This fluid and the degenerating blood-island cells were believed to give rise to the blood plasma. This left a rim of cells around the periphery which differentiated into the vessel endothelium (Sabin, 1917, 1920; Sugiyama, 1926; Houser *et al*, 1961).

Thus, in contrast to the currently accepted descriptions of the direct transformation of the original blood-islands into both the vessel endothelium and intravascular blood cells (see description in Section 1.2.2 above), Sabin, Sugiyama and Houser believed that the yolk sac blood-islands first gave rise to hollow blood vessels which were initially free of intravascular blood cells. According to Sabin (1917, 1920), Sugiyama (1926) and Houser *et al* (1961), intravascular blood-island cells were subsequently produced *de novo* by means of the budding off (by mitotic division) of cells from the endothelium itself into the lumens of the vessels. At first, in view of their alleged method of formation, these newly formed intravascular blood-islands were therefore unicellular. However, they soon divided by mitosis to form multicellular blood-island aggregates within the vessel lumens. According to these authors, these intravascular blood-island cells then differentiated erythropoietically and eventually became free within the vessel lumens as the first developing blood cells (Sabin, 1917, 1920; Houser *et al*, 1961).

Thus, Sabin, Sugiyama and Houser believed that the intravascular blood-islands were formed anew only after the original blood-island aggregates had become transformed into hollow blood vessels. For this reason Sabin (1917, 1920) preferred to call the original blood-island masses the 'angioblasts' and to restrict the term 'blood-islands' to refer to the cells which were subsequently produced *de novo* by the budding off of cells from the vessel endothelium into the lumens of the vessels. The term 'angioblasts' implies that the anlagens are the forerunners of the vessel endothelium (Sabin, 1920).

Although the above method of vasculogenesis was the most frequent method observed in their studies, Sabin (1920), Sugiyama (1926) and Houser *et al* (1961) also found that in a minority of cases the central area of the original blood-islands (angioblasts) did not degenerate completely, but left behind groups of intravascular blood-island cells. In these cases the peripheral cells merely became more elongated than those at the centre and acquired the morphological characteristics of an endothelium. As this occurred, the peripheral cells became separated from the more central cells by intercellular spaces (Houser *et al*. 1961). According to these authors, "Vascular luminization occurs

infrequently in this manner and then only in the larger clumps of angioblasts located in the most posterior portion of the area pellucida." (Houser *et al*, 1961 page 31).

This latter description of blood-island differentiation which Sabin, Sugiyama and Houser *et al* describe as being infrequent is similar to the currently accepted descriptions of yolk sac vasculogenesis given by all the other authors in the field (Murray, 1932; Mato *et al*, 1964; Edmonds, 1966; Gonzalez-Crussi, 1971; Murphy and Carlson, 1978). As indicated in Section 1.2.2 above, these authors describe vessel luminization as occurring without the degeneration of the central cells of the blood-islands: The peripheral cells of the blood-islands become flattened, their nuclei elongate, and they develop into the vessel endothelium (Murray, 1932; Mato *et al*, 1964; Edmonds, 1966; Gonzalez-Crussi, 1971). As this occurs, the developing endothelium becomes separated from the central blood-island cells by the formation of fluid-filled intercellular spaces (Edmonds, 1966; Gonzalez-Crussi, 1971). Meanwhile the central cells of the blood-islands remain intact within the newly formed vessel lumen, and they give rise to the first developing blood cells (Edmonds, 1964, 1966; Gonzalez-Crussi, 1971; Small and Davies, 1972; Murphy and Carlson, 1978). Indeed, a number of TEM studies of yolk sac vasculogenesis have failed to reveal evidence for the lysis of the central blood-island cells; nor do they support an endothelial origin for the intravascular blood-island cells (Edmonds, 1964, 1966; Gonzalez-Crussi, 1971; Murphy and Carlson, 1978).

In view of the discrepancies between the Sabin-Sugiyama-Houser descriptions and the currently accepted descriptions in the field, part of the morphological study carried out in Chapter Two of the present thesis has focused attention on uncovering possible reasons for these different types of interpretations. In the present study observations have been made in both the AOV and the AP, allowing for an integrated analysis of the developmental events in both regions. From this study a possible explanation for the discrepancies between the currently accepted theories and the theories of Sabin, Sugiyama and Houser *et al* has been proposed (see Chapter Two).

1.2.4 The formation of the extraembryonic coelom

At the same time that the blood-islands are differentiating into the yolk sac vascular system, the rest of the cells in the mesodermal sheet become aligned to form the linings of the extraembryonic coelom (see Section 1.2.2). The current interpretation of the method of coelomogenesis in the literature is in line with the description originally provided by Sabin (1920) from her observations in the AP of the living chick blastoderm. According to this author, the mesodermal cells dorsal to the blood-islands (angioblasts) become arranged to form two adjacent layers of cells, a dorsal layer, the forerunner of the somatopleure, and a ventral layer, the forerunner of the splanchnopleure. At first there are no spaces between these two layers. However, isolated clefts gradually appear between the two layers. These clefts then flow together to form the cavity of the coelom.

This description of Sabin (1920) is in line with the present observations of coelomogenesis in the AP (see Section 2.3.3.2, Chapter Two). On the other hand, the method of coelomogenesis in the AOV is found to be slightly different (see Section 2.3.2.2, Chapter Two). However, it is found that the current literature descriptions of coelomogenesis in the AOV (Gonzalez-Crussi, 1971; Murphy and Carlson, 1978) are similar to Sabin's description of coelomogenesis in the AP. The description of Gonzalez-Crussi (1971) is as follows: "By stage 8, however, mesodermal cells dorsal to the blood-islands realigned themselves and delaminated into splanchnopleure and somatopleure. This process took place by confluence of multiple zones of detachment, and once completed, these sheets remained unmistakably distinct through future development." (Gonzalez-Crussi, 1971 page 443). In the description of Murphy and Carlson (1978) these authors state: "Between stages 8 and 9, lateral mesoderm split to form two distinct layers." (Murphy and Carlson, 1978 page 347).

As indicated above, one of the findings in the present study (Sections 2.3.2.2 and 2.3.3.2, Chapter Two) is that the interpretations of Gonzalez-Crussi (1971) and Murphy and Carlson (1978) outline the method of coelomogenesis which occurs in the AP only. It is found that the method of coelomogenesis which occurs in the AOV is slightly different.

In agreement with the description of Sabin (1920) it is found that in the AP the mesodermal cells do indeed become aligned to form two

adjacent layers of cells which then delaminate to form the cavity of the extraembryonic coelom (Section 2.3.3.2, Chapter Two). In the AOV, on the other hand, the coelom does not appear to be formed from the splitting apart of two adjacent layers of cells. Rather, the coelom in the AOV is formed when the mesodermal cells become arranged to form the walls of small blind channels, the lumens of which are the forerunners of the coelom. These small channels coalesce with each other to form larger and larger channels which finally unite to form the large coelomic spaces of the AOV. A description of this morphogenesis is presented in Section 2.3.2.2 (Chapter Two).

1.3 Tissue Interactions during Yolk Sac Vasculogenesis

A common feature of embryonic development is the translocation of cells, or sheets of cells, from one region of the blastoderm to another (see Trinkaus, 1967, 1976; Dieterlen-Lievre, 1978; Le Douarin, 1980). By this means, cells from widely separated areas of the blastoderm may come to lie side by side. A result of this is that new cellular associations may be set up between the different types of cells. These mutual interactions may influence the future developmental histories of these cells.

The centrifugal migration of the mesodermal cells from the primitive streak into the AOV of the chick blastoderm is a typical example of such a situation. As a result of this translocation, the mesodermal cells become associated to a greater or lesser extent with the endoderm and ectoderm of the AP and AOV. Wilt (1965a) and Miura and Wilt (1969) have shown that the opaca endoderm stimulates the mesoderm to differentiate into the yolk sac blood system. Likewise, Bennett (1973) has demonstrated that the mesoderm, in turn, stimulates the opaca endoderm to differentiate into an epithelium. A description of these studies follows in Section 1.3.1 below.

1.3.1 Experimental studies of the influences exerted by the endoderm and mesoderm on each other during the course of their differentiation

The influence of the endoderm on the differentiation of the mesoderm

As indicated in Section 1.2.2 above, the differentiation of the yolk sac vascular system takes place in close association with the yolk sac

endoderm. Indeed, the first overt sign of yolk sac vasculogenesis is the clumping together of the blood-island cells against the yolk sac endoderm.

Wilt (1965a) and Miura and Wilt (1969) made use of *in vitro* culturing techniques to investigate the role of the opaca endoderm in the erythropoietic differentiation of the yolk sac mesoderm. These workers cultured fragments composed of the stage 4 opaca ectoderm-plus-mesoderm either alone or in combination with the opaca endoderm. The explants were cultured either on solid organ culture medium (Wilt, 1965a) or on plasma clots attached to millipore filters (Miura and Wilt, 1969). In the latter method, the endoderm was placed either on the same, or on the opposite, side of the millipore filter (Miura and Wilt, 1969). The results of these experiments indicate that the erythropoietic differentiation of the explants was qualitatively and quantitatively enhanced when the ectoderm-plus-mesodermal fragments were cultured in the presence of the endoderm, compared to when they were cultured alone (Wilt, 1965a; Miura and Wilt, 1969). Furthermore, it was shown that the presence of the endoderm was required for the normal morphogenesis of the blood-islands into both the blood cells and the vessel endothelium (Wilt, 1965a; Miura and Wilt, 1969).

Miura and Wilt (1969) did not consider the action of the endoderm on the opaca ectoderm-plus-mesoderm to be a classical inductive interaction, because the responding tissue still showed some ability to differentiate along erythropoietic lines even in the absence of the endoderm. Rather, they considered the effect of the endoderm on mesodermal erythropoiesis to be "salutary", i.e., it provided a more favourable, or permissive, micromilieu for erythropoiesis (Miura and Wilt, 1969).

The influence of the mesoderm on the differentiation of the endoderm

Just as the yolk sac endoderm has been shown to exert a salutary influence on the differentiation of the yolk sac blood system, so too has it been shown that the developing blood system exerts a permissive influence on the differentiation of the yolk sac endoderm (Bennett, 1973).

Bennett (1973) studied the differentiation of the yolk sac endoderm in the chick blastoderm, using the appearance of the enzyme cysteine lyase as a marker of endodermal cell differentiation. Using histochemical

techniques, this worker found that the differentiation of the opaca endoderm was temporally coupled with the migration of the mesoderm into the AOV and its differentiation into the yolk sac blood system. This was confirmed experimentally by culturing fragments of the opaca ectoderm-plus-endoderm either alone or in combination with the yolk sac blood system or blood cells (Bennett, 1973). Analogously with Wilt's (1965a) and Miura and Wilt's (1969) observations of the salutary effect exerted by the endoderm on the differentiation of the mesoderm, Bennett (1973) found that the mesoderm too exerted a stimulatory, but not essential, effect on the differentiation of the yolk sac endoderm. Thus, while the yolk sac endoderm itself was capable of synthesizing cysteine lyase, the production of cysteine lyase was enhanced when the endoderm was cultured in the presence of the yolk sac blood system or blood cells (Bennett, 1973). Bennett found, furthermore, that the normal morphogenesis of the endoderm into a single-layered epithelium was dependent upon its physical combination with the mesoderm. This effect is analogous with that exerted by the endoderm on the normal morphogenesis of the mesoderm (Wilt, 1965a).

These studies of Wilt (1965a), Miura and Wilt (1969), and Bennett (1973) have demonstrated the mutual interdependence between the yolk sac endoderm and mesoderm during the course of their morphogenesis and cyto-differentiation. The salutary influence exerted by the endoderm on the development of the blood system, and that exerted, in turn, by the blood system on the differentiation of the endoderm, highlights not only the interactive nature of the parts, but also the importance of the spatial arrangements of these parts. In this regard, it is of special interest that the differentiation of the blood-islands occurs in close association with the endoderm and not with the ectoderm (see Figs. 1.1, 1.4). This is of further relevance in view of the finding from the present thesis that the ectoderm itself exerts an inhibitory effect on blood-island differentiation (see Section 4.3.2, Chapter Four).

In addition to this spatial element, the present study has focused attention also on the timing of these interactive events (Sections 4.3.3 and 4.3.4, Chapter Four), concentrating on the temporally and spatially co-ordinated creation of the yolk sac vascular system in both the AP and AOV (see Chapters Two, Three and Four). Since the mesodermal cells which will eventually give rise to the yolk sac vascular system originate in the central axis of the blastoderm (the primitive streak) from where they migrate centrifugally through the AP and AOV (Fig. 1.3), it is relevant to

question why, for example, these migratory cells do not initially stop in the AP and embark upon their overt differentiation there, but rather continue to migrate into the AOV where they subsequently stop migrating and proceed with their overt differentiation (see, for example, Section 4.3.3, Chapter Four).

1.4 The Role of the Extracellular Matrix during Embryogenesis

Not only are cell-cell interactions important during ontogenesis, but the interactions between the cells and the extracellular matrix also appear to play an important role in the processes of morphogenesis and cytodifferentiation (see Manasek, 1975; Hay, 1977; Lash and Vasan, 1977, 1978; Toole *et al*, 1977; Adamson, 1981).

The cells of the embryo are in intimate association with the contents of the extracellular regions, which comprise the immediate environment within which the cells perform their various functions. The components of the extracellular matrix include macromolecules, water and ions (see Manasek, 1975). The major macromolecular components are the glycosaminoglycans, collagens and glycoproteins, which are products of the cells themselves (see Manasek, 1975; Adamson, 1981). The embryonic extracellular environment is dynamic, there being continual changes in its macromolecular composition as the differentiation of the cells themselves proceeds. These molecular changes may alter other physical and/or chemical characteristics of the extracellular matrix, such as its structural properties, degree of hydration, ionic composition, and permeability (see Manasek, 1975; Toole *et al*, 1977). Changes in the extracellular environment may act back upon the cells themselves, modulating their subsequent behaviour and/or pathways of differentiation (see Bernfield and Banerjee, 1972; Bernfield *et al*, 1972, 1973; Manasek, 1975; Hay, 1977; Lash and Vasan, 1977, 1978; Toole *et al*, 1977, Hay blom *et al*, 1979, 1980; Fisher and Solursh, 1979a,b; Pennypacker, 1979, Adamson, 1981). These changes in the cells may bring about further changes in the macromolecular composition of the extracellular environment, and so on.

In the present thesis a preliminary investigation of the question of the role of the extracellular matrix components, hyaluronate and collagen, on blood-island differentiation is undertaken (Chapter Five). In this study, the effect of the enzymatic removal of hyaluronate and collagen on mesodermal erythropoiesis is investigated.

1.5 General Overview of the Present Investigation

The present study takes a broad perspective in analysing the temporally and spatially co-ordinated creation of a three-dimensional pattern of tissue architecture. In studying the cellular events involved in the morphogenesis of the mesodermal cells into the yolk sac vascular system, attention has been focused on the role of the associated germinal layers (the ectoderm and endoderm), in the different regions of the blastoderm (the AP and ACV), and at different stages of morphogenesis (from stage 1-2 to stage 8-9). This study has integrated a descriptive histological approach (Chapter Two) with an experimental approach (Chapters Three to Seven), the latter involving:

1. the transplantation of radioactively labelled mesodermal fragments or blood-island fragments into given regions of host blastoderms of given ages [illegible] and investigating their subsequent developmental fates in these various locations by means of autoradiography of histological serial sections (Chapter Three and Seven);

2. culturing together small fragments of the blastoderm, composed of [illegible] combinations of the germinal layers, in a small drop of liquid culture medium and analysing:

(a) the influence of the ectoderm and endoderm of different ages and from different regions of the blastoderm on mesodermal erythropoiesis (Chapter Four);

(b) the effect of the enzymatic removal of the extracellular matrix components, hyaluronate and collagen, on mesodermal erythropoiesis (Chapter Five);

(c) the effect of physically flattening the explants on mesodermal erythropoiesis (Chapter Six).

In each chapter the results and observations are analysed and discussed in terms of the overall morphogenetic patterning of the various components of the yolk sac vascular system. Finally, in Chapter Eight (the Concluding Discussion), the findings from the previous chapters are synthesized into a broad overview, and some hypotheses are proposed to explain the temporal and spatial elaboration of this three-dimensional pattern.

1.5 General Overview of the Present Investigation

The present study takes a broad perspective in analysing the temporally and spatially co-ordinated creation of a three-dimensional pattern of tissue architecture. In studying the cellular events involved in the morphogenesis of the mesodermal cells into the yolk sac vascular system, attention has been focused on the role of the associated germinal layers (the ectoderm and endoderm), in the different regions of the blastoderm (the AP and AOV), and at different stages of morphogenesis (from stage 1-2 to stage 8-9). This study has integrated a descriptive histological approach (Chapter Two) with an experimental approach (Chapters Three to Seven), the latter involving:

1. the transplantation of radioactively labelled mesodermal fragments or blood-island fragments into given regions of host blastoderms of given ages, and investigating their subsequent developmental fates in these various locations by means of autoradiography of histological serial sections (Chapters Three and Seven),
2. culturing together small fragments of the blastoderm, composed of given combinations of the germinal layers, in a small drop of liquid culture medium and analysing:

(a) the influence of the ectoderm and endoderm of different ages and from different regions of the blastoderm on mesodermal erythropoiesis (Chapter Four);

(b) the effect of the enzymatic removal of the extracellular matrix components, hyaluronate and collagen, on mesodermal erythropoiesis (Chapter Five);

(c) the effect of physically flattening the explants on mesodermal erythropoiesis (Chapter Six).

In each chapter the results and observations are analysed and discussed in terms of the overall morphogenetic patterning of the various components of the yolk sac vascular system. Finally, in Chapter Eight (the Concluding Discussion), the findings from the previous chapters are synthesized into a broad overview, and some hypotheses are proposed to explain the temporal and spatial elaboration of this three-dimensional pattern.

CHAPTER TWO

THE MORPHOGENESIS OF THE CHICK YOLK SAC VASCULAR SYSTEM AND EXTRA-EMBRYONIC COELOM AS STUDIED WITH THE LIGHT- AND SCANNING ELECTRON MICROSCOPES

2.1 Introduction

The present chapter describes the SEM and light microscopic study which was carried out to analyse the changing cellular organization and patterning which occurs during the morphogenesis of the yolk sac vascular system and extraembryonic coelom in the early chick blastoderm. As indicated previously, the morphogenesis of this system involves the transformation of an irregular mass of mesenchymal cells into a spatially ordered pattern of tubes (blood vessels), free cells (intravascular blood cells), and large cell-lined spaces (the extraembryonic coelom) (see Chapter One).

The SEM has proved an excellent tool for investigating cellular patterning because it enables one to visualize immediately the tissue construction in three dimensions. This technique provides one not only with visual information about the shapes, sizes and surface textures of the individual cells, but also produces a lucid picture of the spatial relationships of the component parts to each other, and to the pattern as a whole. The use of light microscopic sections, especially thin 1µm plastic sections, was found to be an invaluable supplement to this SEM information due to the clarity of the individual cell outlines provided by this method.

Many aspects of the present investigation overlap with aspects of previous descriptive studies of yolk sac vasculogenesis which have been carried out using a variety of histological techniques: fixed and stained light microscopic sections (Danchakoff, 1907, 1908), living unstained material (Sabin, 1917, 1920; Houser *et al*, 1961), fixed and stained cultured material (Murray, 1932), TEM sections (Mato *et al*, 1964; Edmonds, 1964, 1966; Gonzalez-Crussi, 1971; Murphy and Carlson, 1978), and SEM sections (Murphy and Carlson, 1978).

The present observations of the cellular events involved in yolk sac vasculogenesis are essentially in agreement with the currently

accepted descriptions in the literature (see Section 1.2.2), but moreover, have also filled in some of the gaps in these descriptions. The present analysis has also provided a possible explanation for the discrepancies which have stemmed from some of the older literature (Sabin, 1917, 1920; Sugiyama, 1926; Houser *et al*, 1961 - see Section 1.2.3).

2.2 Materials and Methods

2.2.1 · Eggs and embryos

Freshly laid fertilized eggs of the Ross Breed (Cornish ♀♀ x White Rock ♂♂) were obtained from Sunnyside Pedigree Poultry (Farm Fare, Pty., Ltd.) or Palala Chix (Farm Fare, Pty., Ltd.). The eggs were incubated at 38°C in a Gallenkamp model IH-100 incubator for the desired period of time.

Chick blastoderms of stages 4 to 14 were dissected from the yolk in chick embryo saline (0,7% NaCl (Analar); 0,037% KCl (Analar); 0,018% $CaCl_2$ (Analar)), and prepared for microscopic examination as follows:

2.2.2 Preparation of tissue for scanning electron microscopy

Chick blastoderms of stages 4 to 14 were fixed overnight at 4°C in 2,5% glutaraldehyde (BDH) made up in phosphate buffer (41,5ml of 2,26% $NaH_2PO_4.H_2O$; 8,5ml of 2,52% NaOH), and thereafter washed in phosphate buffer. Using a sharp blade, the blastoderms were cut transversely into 2mm strips, leaving a lateral edge of the blastoderm intact so that the sections remained in position. The blastoderms were attached by means of their intact lateral edge to a piece of aluminium foil (Fig. 2.1) on which they were supported during subsequent processing.

The blastoderms were post-fixed for 35 mins at room temperature in 1% osmium tetroxide (EMscope) made up in phosphate buffer, washed in deionized water, dehydrated through a graded series of ethanol, and the ethanol replaced with amyl acetate. The blastoderms were critically point dried in a Bomar SPC-900/EX critical point drying apparatus, using carbon dioxide as the transition fluid.



Figure 2.1

Diagram of a blastoderm cut into 2mm strips and supported on aluminium foil for handling during processing.

The blastoderm sections were removed individually from the aluminium foil, and mounted with colloidal graphite (Acheson Colloids Company, Plymouth) on aluminium stubs. The specimens were either coated with carbon and gold palladium, or carbon and gold, in an Edwards Pirani Penning model 4 coating unit, or were sputter coated with gold palladium in a Hummer II sputter coater. The sections were examined in a Cambridge S4 Stereoscan electron microscope operating at 20-30 KV.

2.2 Preparation of tissue for light microscopy

Three types of tissue preparations were employed for the light microscopic examinations of the blastoderms:

1. wax embedded serial cross sections (Section 2.2.3.1),
2. plastic embedded sections (Section 2.2.3.2), and
3. whole-mounted blastoderms (Section 2.2.3.3).

2.2.3.1 Preparation of wax embedded serial cross sections

Chick blastoderms of stages 4 to 14 were fixed for 24 hours at $4^{o}C$ in Smith's fixative (Humason, 1967), washed overnight in running tap water, dehydrated through a graded series of ethanol, cleared in methyl benzoate, and embedded in Paraplast (Sherwood Medical Industries, St. Louis). The blastoderms were serially sectioned at 4-7μm on a model 820 Spencer microtome. The ribbons were mounted, without adhesive, on precleaned glass slides. Following deceration in xylol and hydration

through a graded series of ethanol, the sections were stained with Mayer's haematoxylin and eosin (Humason, 1967), dehydrated through ethanol, cleared in xylol, and coverslips were applied to the slides with DPX mountant.

2.2.3.2 Preparation of plastic embedded sections

Chick blastoderms of stages 8 and 11 were fixed for 2,5 hours at room temperature in 'TAG' (Rodewald and Karnovsky, 1974) made up of 1% tannic acid (Schering-Kahlbaum A.G., Berlin) and 1% glutaraldehyde (BDH) in phosphate buffer. The blastoderms were then washed in phosphate buffer. 1mm x 1mm fragments were excised from the AOV with a sharp blade, and were post-fixed for 30 minutes at room temperature in 1% osmium tetroxide (EMscope) made up in phosphate buffer. Thereafter the tissue fragments were washed in deionized water, stained in block in 1% uranyl acetate made up in 70% acetone. dehydrated in acetone, cleared in propylene oxide, and embedded in Spurr resin (Polaron). Polymerization was at 70°C for 10 hours.

Sections of 1µm were cut with glass knives on a Porter Blum MT-2 ultra-microtome and mounted on precleaned glass slides. The sections were stained with toluidine blue (see Mercer and Birbeck, 1972), air-dried overnight, and coverslips were mounted with DPX.

2.2.3.3 Preparation of whole-mounted blastoderms

Chick blastoderms of stages 4 to 14 were fixed for 24 hours at 4°C in Smith's fixative (Humason, 1967), washed overnight in running tap water, stained with Mayer's haematoxylin (Humason, 1967), dehydrated through a graded series of ethanol, cleared in xylol, and placed on precleaned glass slides. A rim of celluloid was placed around the periphery of each blastoderm to form an even support for the coverslips, which were then mounted with DPX.

2.3 Observations

2.3.1 The origin and migration of the primary mesenchymal cells

The mesodermal cells which give rise to the yolk sac vascular system and extraembryonic coelom originate in the epiblast (Rosenquist, 1966). From stage 2 (Hamburger and Hamilton, 1951) these cells begin

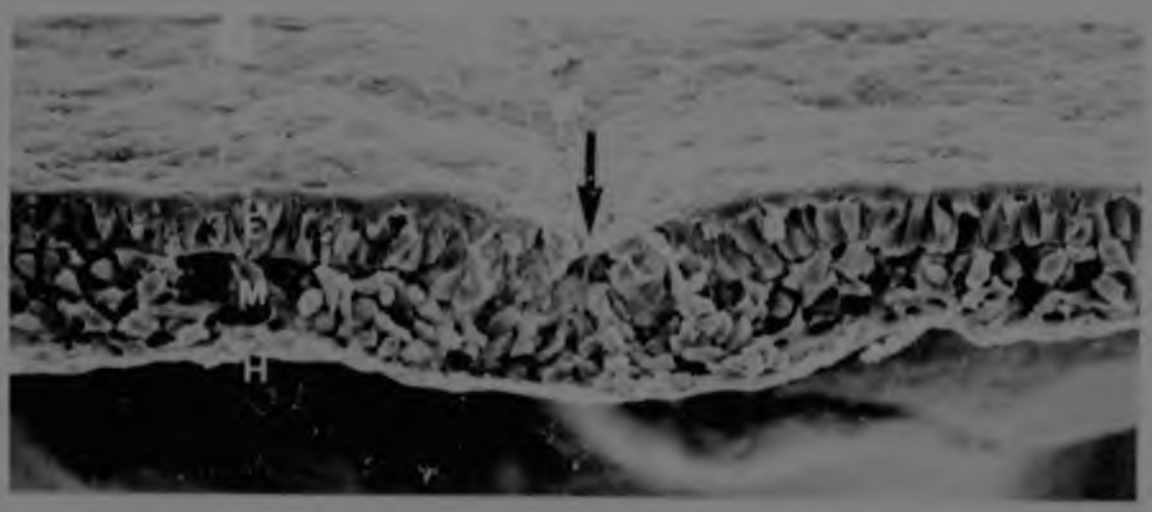


Figure 2.2

Scanning electron micrograph of a stage 4 chick blastoderm in the region of the primitive streak (arrow). The cells from the epiblast (E) invaginate through the primitive streak into the space between the epiblast (E) and hypoblast (H). These cells become the primary mesenchymal cells (M).

E, epiblast; H, hypoblast; M, primary mesenchymal cells; arrow, primitive streak.

x 446

to migrate through the primitive streak into the space between the epiblast and hypoblast (Figs. 2.2 to 2.4). Balinsky and Walther (1961) and Granholm and Baker (1970), in TEM studies of chick gastrulation, drew attention to the role of cell-shape in the invagination of the primitive streak cells. Thus, whereas the epiblast lateral to the primitive streak is in the form of a simple columnar epithelium (Figs. 2.2, 2.5), in the region of the primitive streak the epiblast is seen to have become multilayered, and its cells are seen to have become progressively more elongated and flask-shaped (Fig. 2.3). As described by Balinsky and Walther (1961), Granholm and Baker (1970) and Solursh and Revel (1978), the apical ends of the flask cells are seen to be constricted, and they form long narrow bottle-necks which maintain their attachments at the surface of the epiblast (Fig. 2.3). The basal ends of the flask cells



Figure 2.3

Scanning electron micrograph of the primitive streak (arrow) of a stage 4 blastoderm. The epiblast (E) is seen to be multilayered. Its cells are seen to have become progressively more elongated and flask-shaped (f) as they migrate into the space between the epiblast (E) and hypoblast (H). Numerous processes occur between adjacent cells, by means of which they make contact with each other.

E, epiblast; f, flask-shaped cells; H, hypoblast; M, primary mesenchymal cells; arrow, primitive streak.

x 1092

are seen to have become expanded, and they extend down into the interior of the blastoderm (Fig. 2.3). Adjacent flask cells are seen to make contact with each other by means of lateral processes (Fig. 2.3), as described by these previous authors.

When the flask cells finally lose contact with the surface of the epiblast, they are seen to have become totally displaced into the space between the epiblast and hypoblast (Fig. 2.3). These cells are seen to have become irregularly shaped, and typically mesenchymal in appearance (Figs. 2.2, 2.3). In this manner the epiblast cells from the surface of the blastoderm become transformed into the primary

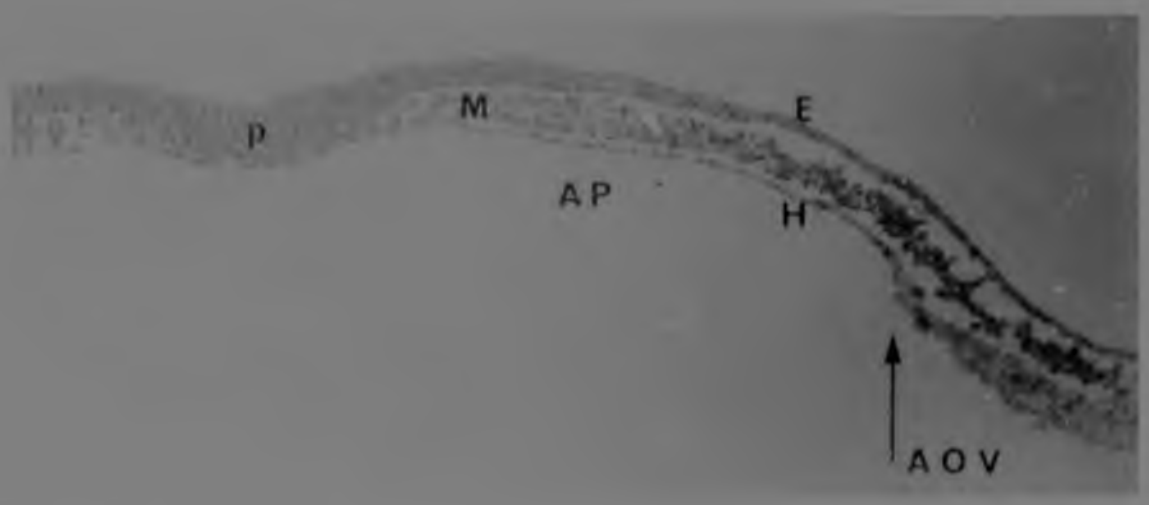


Figure 2.4

Light micrograph of a 4-7µm wax-embedded section through a stage 5 chick blastoderm. The primary mesenchymal cells (M) which invaginate through the primitive streak (p) form an irregular sheet of cells which spreads through the area pellucida (AP) and the vasculosa part of the area opaca (AOV). The border region between the AP and AOV is indicated with an arrow.

AOV, area opaca vasculosa; AP, area pellucida; E, epiblast; H, hypoblast; M, primary mesenchymal cells; p, primitive streak; arrow, border region between the AP and AOV.

x 208

mesenchymal cells (mesoblast) in the interior of the blastoderm (Balinsky and Walther, 1961; Granholm and Baker, 1970; Solursh and Revel, 1978).

The primary mesenchymal cells form a loose irregular network of cells in the space between the epiblast and hypoblast (Figs. 2.2 to 2.5). Large intercellular spaces occur between adjacent mesenchymal cells, and between the mesenchymal cells and the overlying epiblastic- and underlying hypoblastic layers (Figs. 2.2 to 2.5). However, the individual mesenchymal cells continue to make contact with each other and with the epiblastic- and hypoblastic layers by means of their cellular processes (Figs. 2.2, 2.3, 2.5), as found by Balinsky and Walther (1961), Trelstad et al. (1967), Granholm and Baker (1970) and Revel and Solursh (1978). In agreement with these authors there is therefore seen to be a physical continuity from the epiblast through the cells of the

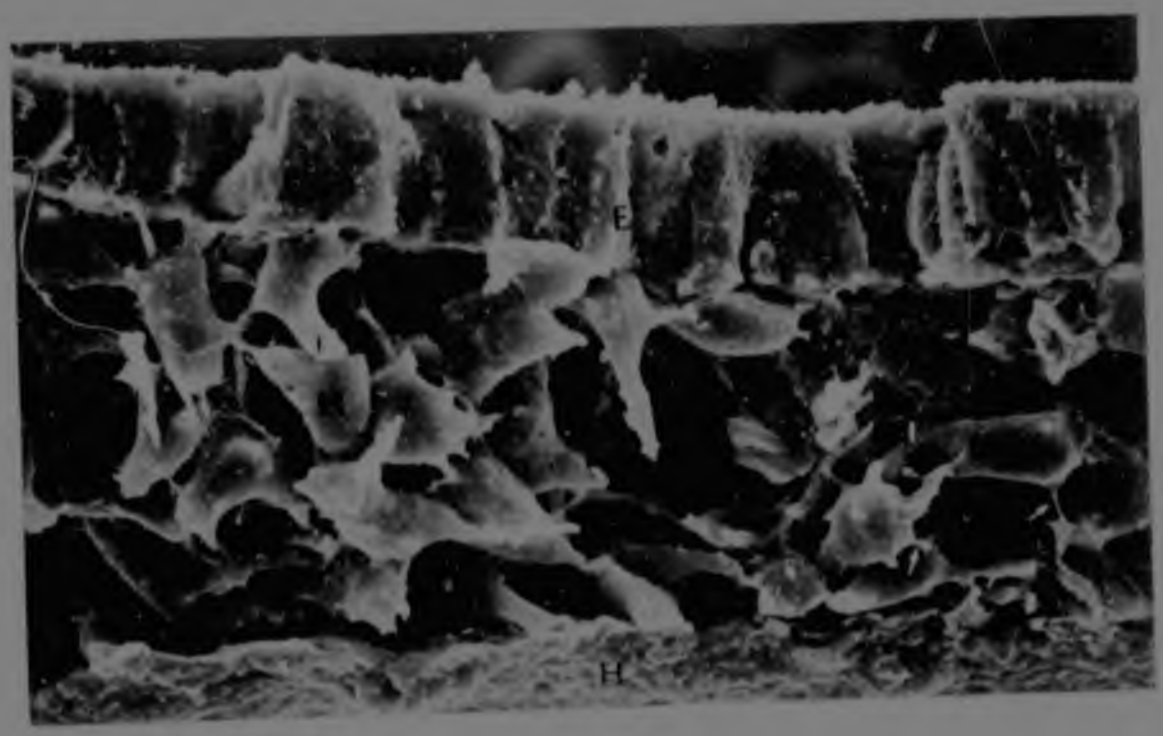


Figure 2.5

Scanning electron micrograph to show the primary mesenchymal cells (M) in the AP lateral to the primitive streak of a stage 4 blastoderm. The primary mesenchymal cells (M) form a loose irregular meshwork in the space between the epiblast (E) and hypoblast (H), there being large intercellular spaces between adjacent cells. The individual cells contact neighbouring mesenchymal cells, as well as the overlying epiblast and underlying hypoblast by means of filopodia (arrows) and lamellipodia (l).

E, epiblast; H, hypoblast; l, lamellipodia; M, primary mesenchymal cells; arrows, filopodia.

x 1638

primitive streak to the primary mesenchyme and to the underlying hypoblast by means of these cellular processes.

In accordance with the SEM observations of Ebendal (1976), Wakely and England (1977) and Revel and Solursh (1978), the cellular processes of the primary mesenchymal cells are seen to be mainly in the form of narrow finger-like projections or filopodia, and broader cytoplasmic extensions or lamellipodia (Figs. 2.5, 2.7). These structures are commonly associated with the active cellular translocations

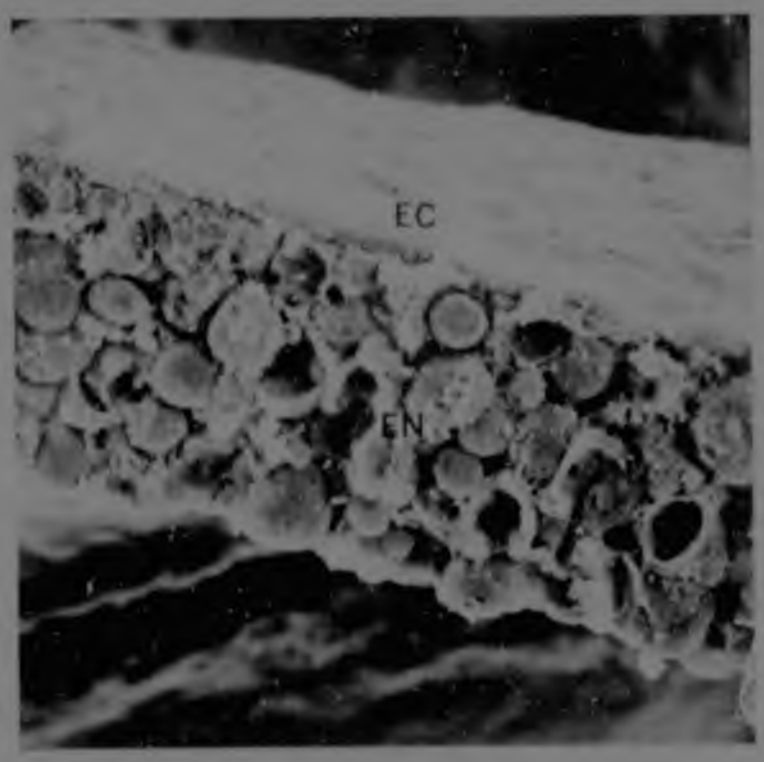


Figure 2.6

Scanning electron micrograph of a cross section through the area opaca vitellina of a stage 5-6 chick blastoderm. The epiblast (which is later called the ectoderm) (EC) is a simple squamous epithelium, while the hypoblast (which is later called the endoderm) (EN) is a thick stratified epithelium composed of large yolk-filled cells. In this region of the blastoderm the ectoderm and endoderm remain closely apposed to each other and do not become separated by extracellular spaces or invaded by the expanding mesodermal sheet.

EC, ectoderm; EN, endoderm.

x 1940

(see Trelstad *et al*, 1967; Hay, 1968; Trinkaus, 1969, 1976; Revel and Solursh, 1978; Abercrombie, 1980). Indeed, the cells from the primitive streak are cells in motion, moving from the epiblast into the mesenchymal layer below, and then centrifugally in the space between the epiblast and hypoblast, forming an expanding sheet of cells which spreads through the AP into the AOV (see Balinsky, 1981). By stage 5 these cells can therefore be seen as an irregular sheet of cells in the AP and AOV (Fig. 2.4).

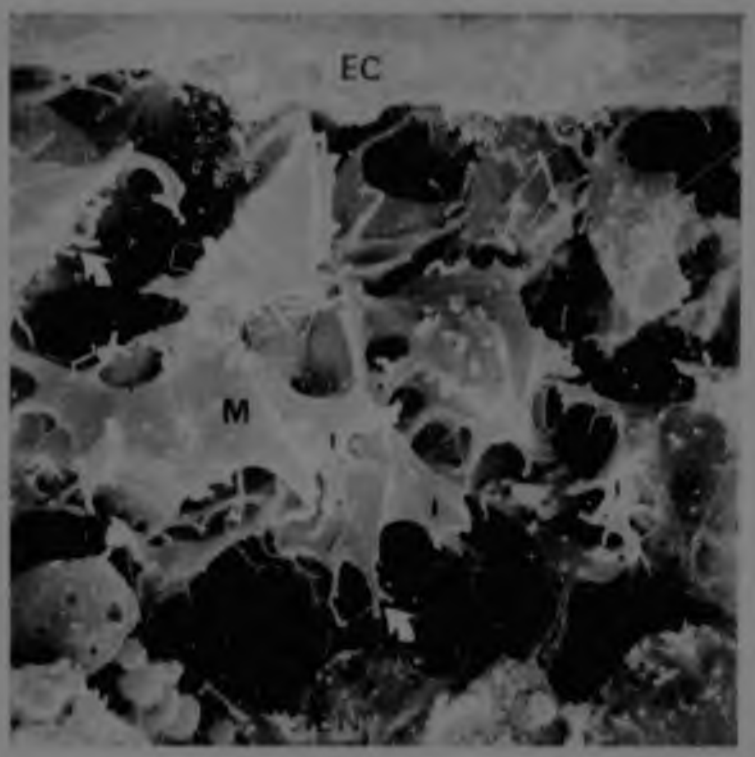


Figure 2.7

Scanning electron micrograph to show the migratory mesenchymal cells (M) which have entered the AOV of a stage 5 blastoderm. The mesenchymal cells (M) are still irregularly shaped and typically mesenchymal in appearance. Numerous filopodia (arrows) and lamellipodia (l) extend from their surfaces, by means of which they make contact with adjacent mesenchymal cells, and with the overlying ectodermal- (EC) and underlying endodermal (EN) layers.

EC, ectoderm; EN, endoderm; l, lamellipodia; M, mesodermal cells; arrows, filopodia.

x 5044

The transition zone between the AP and AOV can clearly been seen in Fig. 2.4, as indicated from the changing appearance of the hypoblastic layer. Thus, whereas the hypoblast in the AP is a simple squamous epithelium, the hypoblast in the AOV is a thick stratified epithelium composed of large yolk-filled cells.

Large yolky hypoblastic cells are also characteristic of the vitellina part of the area opaca (Fig. 2.6) which borders the AOV distally. As indicated previously (Section 1.2.2), the boundary between the vasculosa- and vitellina parts of the area opaca is

established by the extent of the migration of the mesenchymal cells into the area opaca (see Patten and Carlson, 1974). The vasculosa region of the area opaca derives its name from the fact that the mesenchymal cells which migrate into this region will differentiate into the yolk sac vascular system. In the distal region of the area opaca (the area opaca vitellina) the epiblast and hypoblast remain closely apposed to each other and do not become separated by cell-free spaces or invaded by the expanding mesenchymal sheet (Fig. 2.6).

The Origin of the Embryonic Endoderm

It should be noted that some of the invaginating cells from the ive streak do not contribute to the mesodermal layer, but enter e underlying hypoblastic layer of the AP where they give rise embryonic endoderm (Hunt, 1937; Rosenquist, 1966; Nicolet, 1

As found by Balinsky and Walther (1961), the entry of these primary mesenchymal cells into the hypoblast is difficult to recognise with certainty in fixed histological material (see Fig. 2.3). Furthermore, as found by Wakely and England (1977), it is difficult to distinguish histologically between the presumptive endodermal- and presumptive mesodermal cells (see Fig. 2.3). However, the tissue transplantation experiments carried out by Rosenquist (1966) and Nicolet (1967, 1970) in which the developmental fates of radioactively labelled blastoderm grafts were followed autoradiographically, clearly demonstrate that some of the primary mesenchymal cells from the primitive streak enter into the endodermal layer. These studies show that this occurs during stages 2 to 4. As the presumptive endodermal cells enter into the endodermal layer, the original hypoblastic cells of the AP become displaced towards the periphery of the AP, adjacent to the hypoblast of the AOV.

From about stage 5-6 onwards it is usual to refer to the lower layer of the blastoderm (in both the AP and AOV) as the endoderm, even though the true endoderm is located only in AP, and the original hypoblastic cells persist in the AOV. It is also usual to refer to the upper layer of the blastoderm as the ectoderm rather than the

epiblast, even though its cells are still invaginating through the primitive streak.

2.3.2 The cellular events involved in vasculogenesis and coelomogenesis in the area opaca vasculosa

As indicated above, the mesodermal cells which migrate into the AOV are destined to differentiate into the yolk sac vascular system (Settle, 1954; Rosenquist, 1966). When these cells first enter into the AOV (stages 4 and 5) they are seen to be still typically mesenchymal in appearance, with numerous filopodia and lamellipodia projecting from their surfaces (Fig. 2.7). By means of these processes, the mesenchymal cells continue to make contact with neighbouring mesenchymal cells, and with the overlying epiblast (ectoderm) and underlying hypoblast (endoderm) (Fig. 2.7).

In agreement with previous authors (see Section 1.2.2), the first visible sign of the differentiation of these cells into the yolk sac vascular system is observable in the posterior and posterio-lateral regions of the AOV at stages 5 and 6. Shortly thereafter the mesodermal cells in the anterior and more medial parts of the AOV are seen to embark on their overt differentiation. A little later, at stage 7+, the mesodermal cells in the AP also start to develop into the vascular system. For this reason it is possible to study a number of successive stages of yolk sac vasculogenesis and coelomogenesis in a single blastoderm. As found by Sabin (1920), different blastoderms of the same stage may vary with respect to the degree of development of the yolk sac vascular system.

The present description of the cellular events involved in the organization of the primary mesenchymal sheet in the AOV into the cellular architecture of the yolk sac vascular system is dealt with under the following headings:

1. The Transformation of Some of the Mesodermal Cells into Yolk Sac Blood-Islands;
2. The Organization of the Rest of the Mesenchymal Cells into the Linings of the Extraembryonic Coelom;
3. The Transformation of the Peripheral Cells of the Blood-Islands into the Endothelial Linings;

4. The Joining up of the Blood-Islands into a Plexus;
5. Further Stages in the Morphogenesis of the Blood Vessels and Intravascular Blood-Island Cells;
6. The Dissolution of the Intravascular Blood-Island Cells;
7. Further Observations of the Yolk Sac Blood Vessels;
8. Further Observations of the linings of the Extraembryonic Coelom;
9. General.

The description of the cellular events of vasculogenesis and coelomogenesis in the AP is dealt with in Section 2.3.3

2.3.2.1 The transformation of some of the mesodermal cells into the yolk sac blood-islands

As indicated above, the first visible sign of yolk sac vasculogenesis is apparent in the posterior and posterio-lateral regions of the AOV at stages 5 to 6. During this period the SEM reveals that some of the primary mesenchymal cells have become transformed into swollen, rough-walled cells which are seen to aggregate together (Fig. 2.8). These aggregating cells give rise to solid cellular condensations of various sizes (Figs. 2.9, 2.10), which are the blood-islands. As described by the previous authors in the field (see Section 1.2.2), the blood-islands are seen to develop in juxtaposition to the yolk sac endoderm (Figs. 2.9, 2.10).

Under the SEM the cells constituting the blood-islands appear as swollen, rough-walled cells which have totally withdrawn their filopodia and lamellipodia and have become very closely packed together (Fig. 2.9). Indeed, the individual cells of the blood-islands are so closely packed together that Sabin (1920) described them as appearing syncytial. Even in the present SEM and wax embedded light microscopic sections it is difficult to discern clearly the individual cell outlines (Figs. 2.9, 2.10). However, it has been clearly demonstrated under the TEM that the individual cells of the blood-islands are discrete from each other (Edmonds, 1964, 1966; Gonzalez-Crussi, 1971).

(Text continues on page 34).

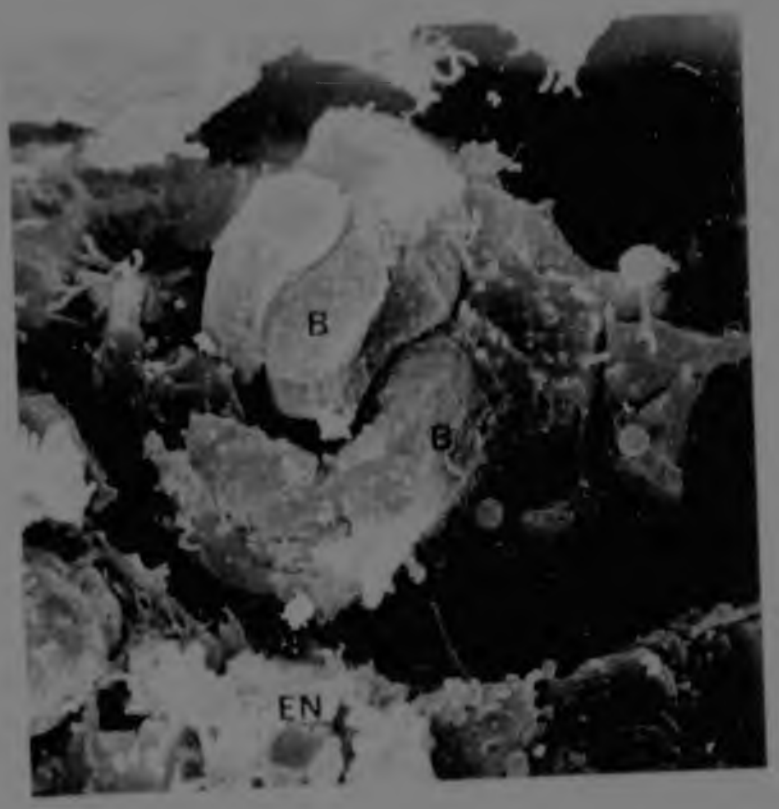


Figure 2.8

Scanning electron micrograph of the area opaca vasculosa of a stage 6 blastoderm. Some of the mesenchymal cells in the sheet are seen to have become transformed into swollen, rough-walled cells (B) which appear to be in the process of aggregating together. These cellular aggregations will give rise to the blood-islands.

B, aggregating blood-island cells; EN, endoderm.

x 3880

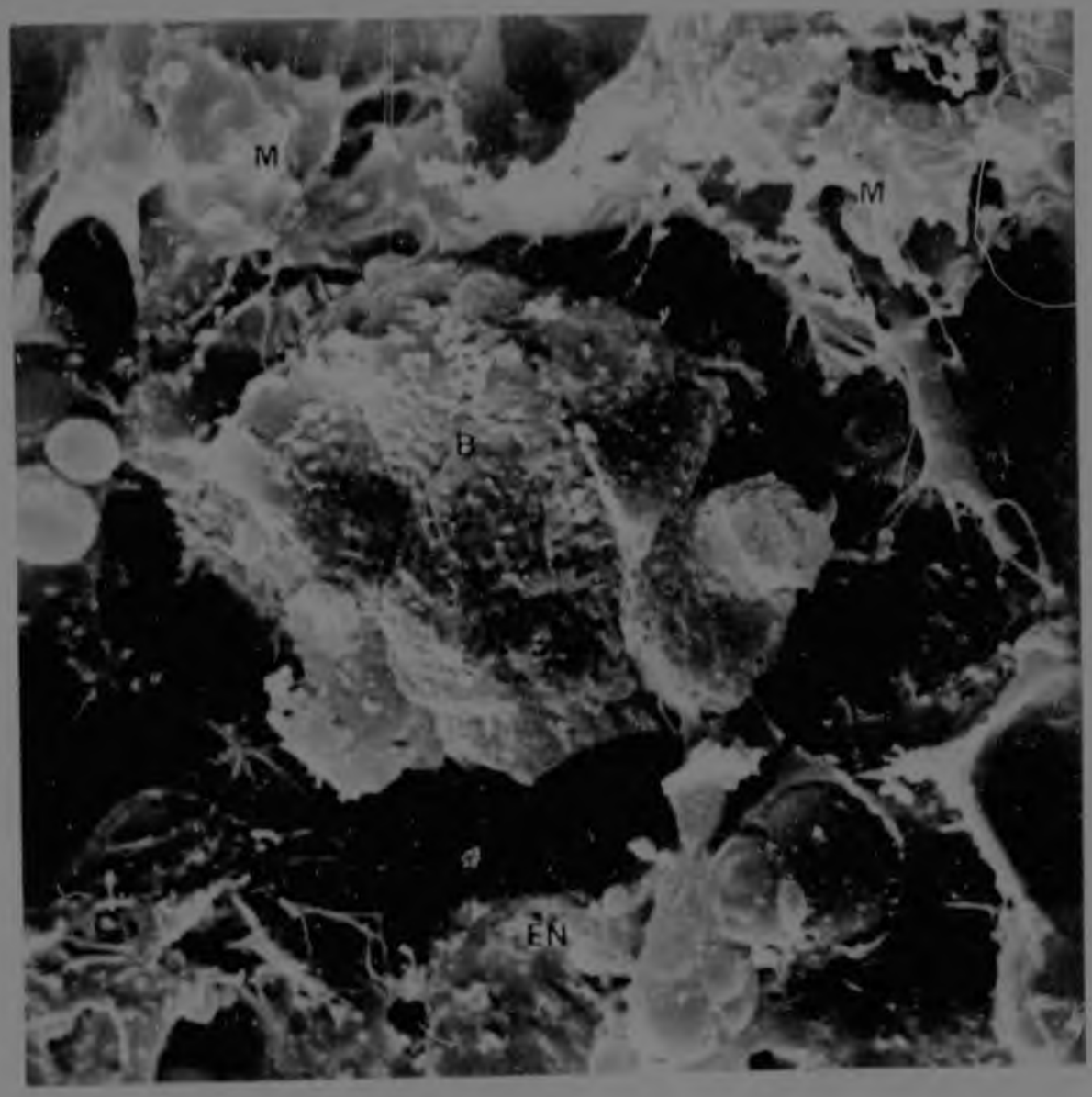


Figure 2.9

Scanning electron micrograph of the area opaca vasculosa of a stage 7 blastoderm. Some of the mesodermal cells in the sheet have clumped together to form a solid cellular aggregation, the blood-island (B). The cells constituting the blood-island appear as swollen, rough-walled cells which are very closely packed together. The rest of the mesodermal cells in the sheet are still mesenchymal in appearance (M). To the right of the blood-island is a primordial germ cell (G) - (see Section 2.3.5).

B, blood-island cells; EN, endoderm; G, primordial germ cell; M, mesenchymal cells.

x 6510

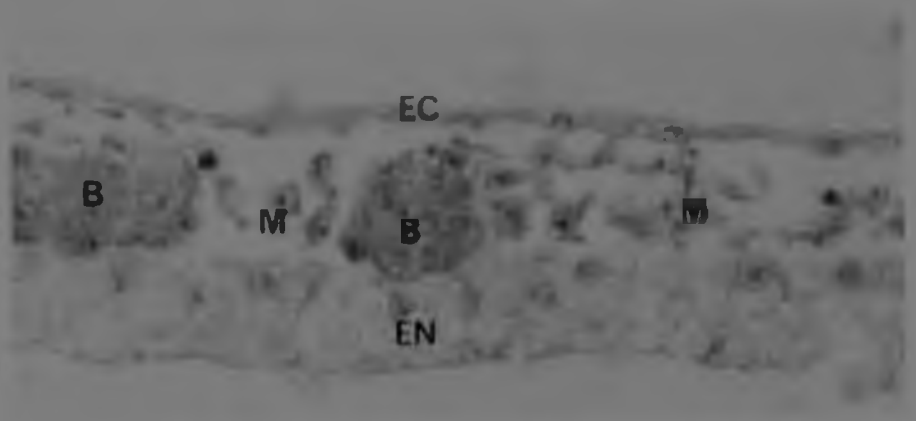


Figure 2.10

Light micrograph of a 4-7μm wax embedded section through the area opaca vasculosa of a stage 6-7 blastoderm. Some of the mesodermal cells in the sheet have clumped together to form solid cellular aggregations of various sizes, the blood-islands (B). The rest of the mesodermal cells on the sheet remain mesenchymal in appearance (M) and they form a loose network of cells in the space above and between the blood-islands.

B, blood-islands; EC, ectoderm; EN, endoderm;
M, mesenchymal cells.

x 667

2.3.2.2 The organization of the rest of the mesenchymal cells into the linings of the extraembryonic coelom

Shortly after the blood-island cells start to clump together against the yolk sac endoderm, the rest of the cells in the mesodermal sheet start to become aligned to form the linings of the extraembryonic coelom. As indicated in Section 1.2.4, the present observations of the cellular events involved in coelom formation in the AOV are not in total accordance with the descriptions of coelomogenesis in the AOV provided by the previous workers in the field (see Gonzalez-Crussi, 1971; Murphy and Carlson, 1978).

In the present study it is found that in the AOV the mesenchymal cells do not become aligned to form two layers of cells which then delaminate, or split apart, to form the coelomic spaces. Rather, the coelom is formed when the mesenchymal cells in the AOV become



Figure 2.11

Scanning electron micrograph of the area opaca vasculosa of a stage 7-8 blastoderm. Some of the mesodermal cells in the sheet have clumped together to form the blood-islands (B). The rest of the cells in the sheet are still typically mesenchymal in appearance (M), with numerous processes extending from their surfaces. These mesenchymal cells form a loose irregular meshwork around the blood-islands in the space between the ectoderm and endoderm (EN).

B, blood-island cells; EN, endoderm; M, mesenchymal cells.

x 4443

arranged to form the walls of small blind channels. These small channels then coalesce with each other to form larger and larger channels. Finally the coalescing channels unite to form the large coelomic spaces of the AOV. A description of the cellular events involved in this morphogenesis is as follows:

At the time during which the blood-island cells are first seen to clump together against the yolk sac endoderm (stage 5 to 6), the rest of the mesodermal cells in the sheet are still mesenchymal

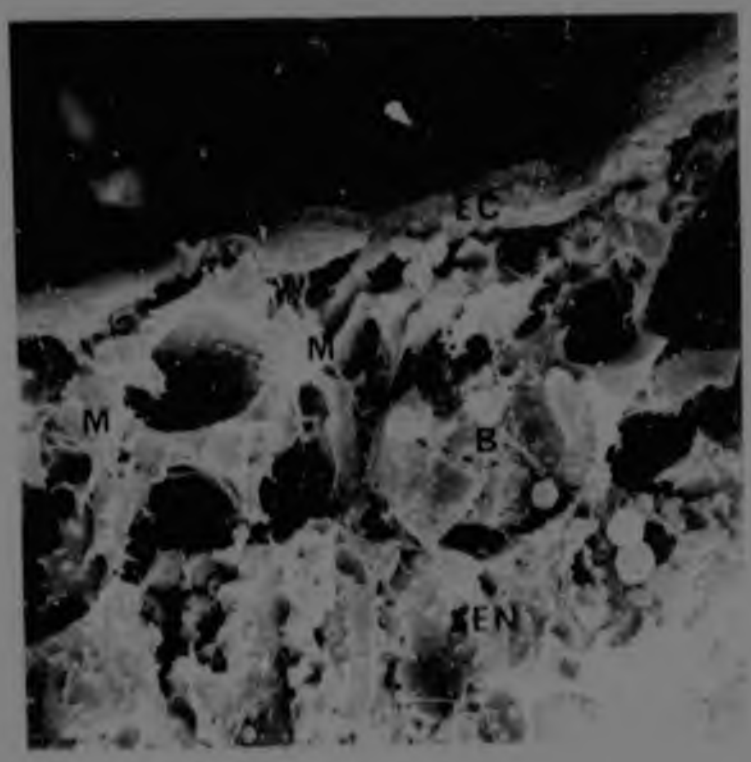


Figure 2.12

Scanning electron micrograph of the area opaca vasculosa of a stage 6-7 chick blastoderm. Some of the mesenchymal cells (M) in the space around the blood-island (B) are seen to have become aligned to form the walls of a small blind channel (C).

B, blood-island; C, small blind channel; EC, ectoderm; EN, endoderm; M, mesenchymal cells.

x 2522

in appearance. These cells form a loose irregular meshwork around the developing blood-islands in the space between the ectoderm and endoderm (Figs. 2.9 to 2.11). In contrast to the closely packed blood-island cells, these mesenchymal cells are still loosely packed with large intercellular spaces between them (Figs. 2.9 to 2.11). Numerous long and short filopodia and lamellipodia project from their surfaces, by means of which they continue to make contact with each other, and with the surfaces of the blood-islands, the endoderm and the ectoderm (Figs. 2.9 to 2.11).

(Text continues on page 38).



Figure 2.13A

Scanning electron micrograph of the endoderm (EN) and mesoderm (M and C) in the area opaca vasculosa of a stage 7 blastoderm. The mesenchymal cells (M) are seen to have become aligned to form a cellular lining (C). The individual cells of the lining are seen to be broad, flat and smooth-walled. Numerous short processes remain along the joins between adjacent cells (arrows), marking the boundaries between the cells. Besides these short processes, the cells tend to have a single longer process (arrowhead) (which may be a cilium - see text page 42) which projects into the lumen of the channel.

C, cells forming a cellular lining; EN, endoderm; M, mesenchymal cells; arrow, short processes between adjacent cells; arrowhead, longer process.

x 6510



Figure 2.13B

Scanning electron micrograph of the area opaca vasculosa of a stage 7 blastoderm. The mesenchymal cells (M) are seen to have become aligned to form the lining (C) of a small channel. The individual cells of the lining are seen to be broad, flat and smooth-walled. Numerous short processes remain along the joins between adjacent cells (arrows), marking the boundaries between the cells. Besides these short processes, the cells tend to have a single longer process (arrow-heads) which projects into the lumen of the channel.

C, cells forming the channel lining; EC, ectoderm;
M, mesenchymal cells; arrow, short processes;
arrow-head, longer process.

x 5005

During stages 6 to 8 these mesenchymal cells are seen to become aligned to form the walls of small blind channels (Figs. 2.12, 2.13A, 2.13B). The process of cellular alignment appears to involve mutual interactions between neighbouring mesenchymal cells (Fig. 2.14). During these interactions, the filopodia along adjacent surfaces of neighbouring cells are seen to become interlaced with each other, and

(Text continues on page 42).

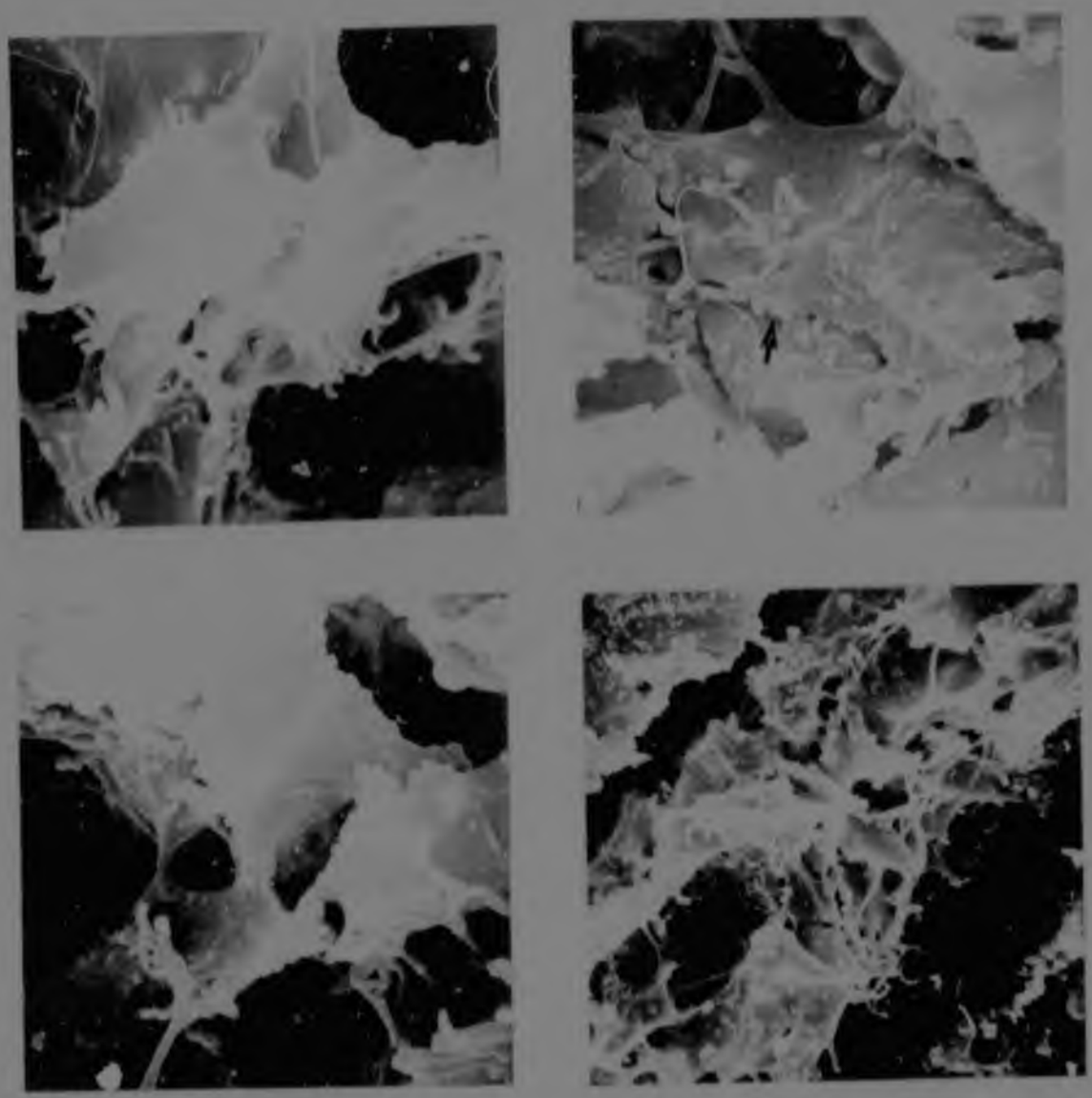


Figure 2.14

Scanning electron micrographs of the mesenchymal cells in the area opaca vasculosa of chick blastoderms of stages 6 to 8. The process of cellular alignment appears to involve the mutual interaction between adjacent mesenchymal cells by means of their cellular processes (arrows). During these interactions, the filopodia along adjacent surfaces of neighbouring cells are seen to become interlaced with each other, and the cells become orientated with their lateral surfaces apposed to each other.

Top left x 7245; Top right x 7262;

Bottom left x 8280; Bottom right x 2898.

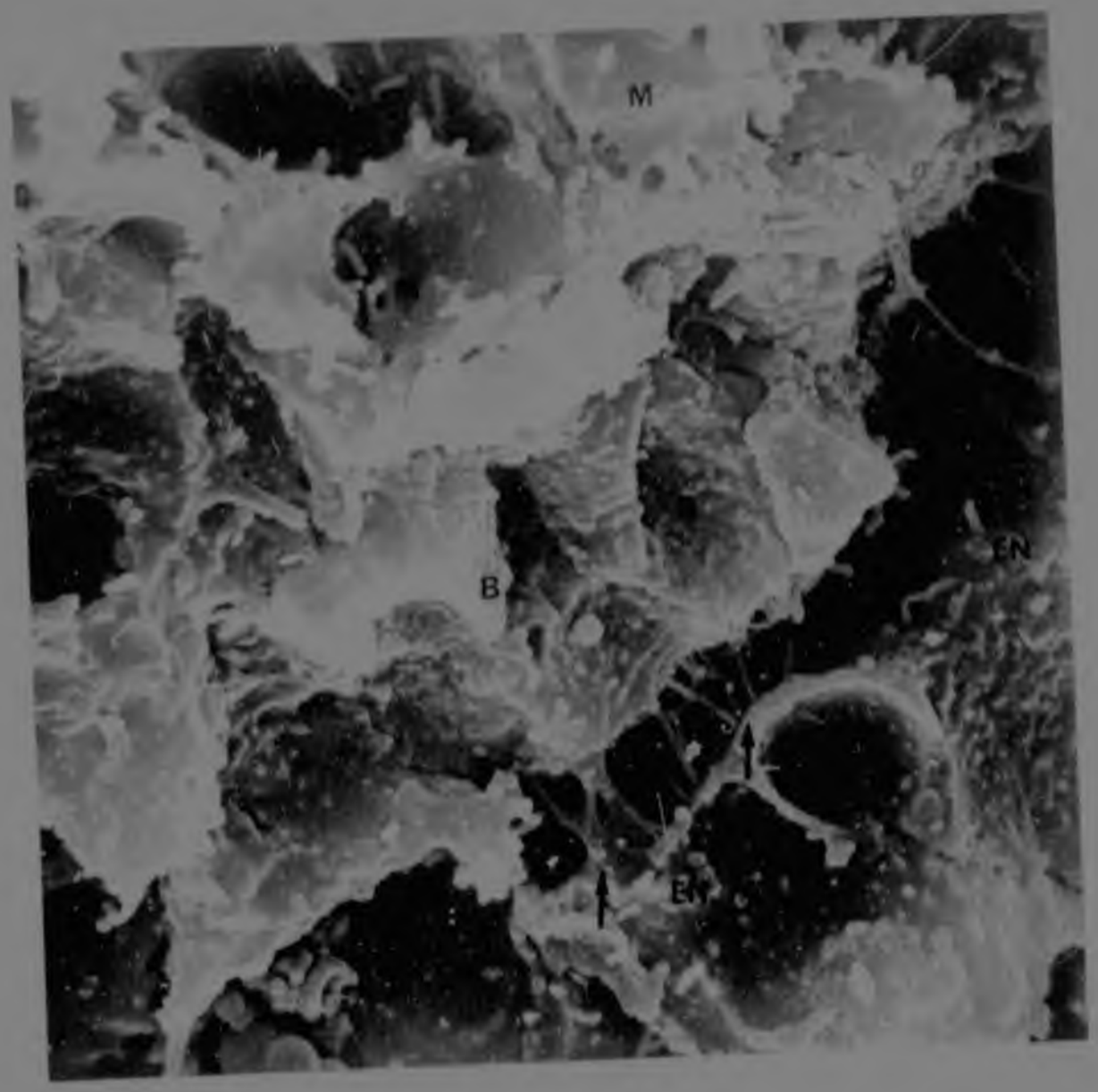


Figure 2.15

Scanning electron micrograph of the area opaca vasculosa of a stage 7 to 8 chick blastoderm. The blood-island cells (B) are seen to be in direct contact with the underlying endoderm (EN) by means of long narrow processes (arrows) which span the space between the blood-island and the endoderm.

B, blood-island cells; EN, endoderm;
M, mesenchymal cells; arrows, long narrow processes.

x 6220



Figure 3.11

Transmission electrograph of the area opaca-vasculosa of 3 somite stage chick blastoderm. The blood-island cells (BI) are seen to be in direct contact with the underlying endoderm (EN) by means of long narrow processes (arrows) which span the space between the blood-islands and the endoderm.

B, blood-island cells; EN, endoderm.
M, mesodermal cells; arrows, long narrow processes.

x 4610

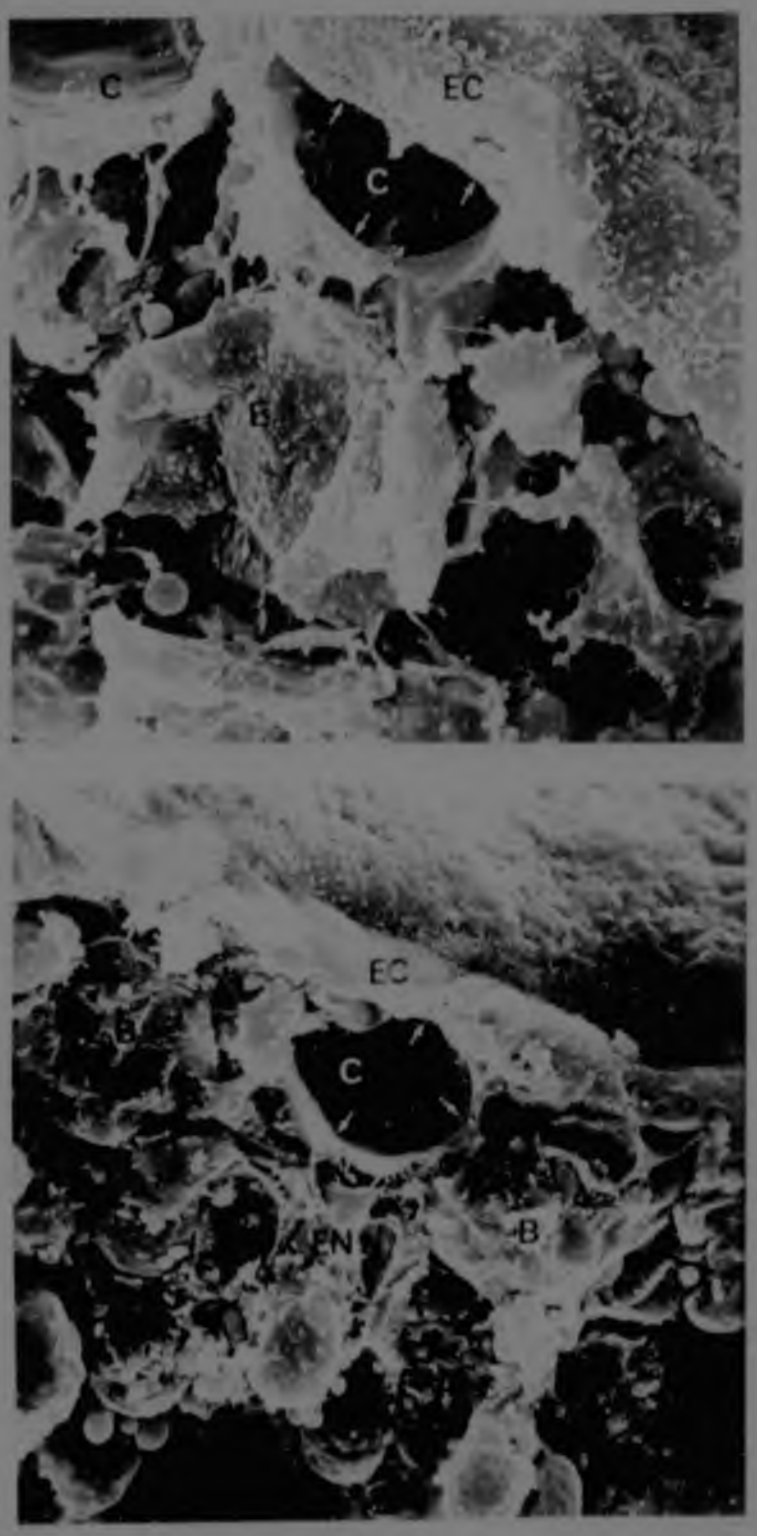


Figure 2.16

Scanning electron micrograph of the area opaca vasculosa of a stage 7-8 chick blastoderm. As the mesenchymal cells become aligned to form the channel linings (C), the outer (i.e. non-luminal) surfaces are seen to maintain contact (arrows) with the blood-islands (B), the endoderm (EN) and the ectoderm (EC), and they are seen to form cellular linings over these surfaces (arrows).

B, blood-islands; C, channel linings; EC, ectoderm; EN, endoderm.

Top x 4074; Bottom x 2000.

the cells are seen to become o ientated witn their lateral surfaces apposed to each other (Fig. 2.14). In this manner, adjacent mesenchymal cells become orientated to form a smooth continuous cellular lining around a central lumen (Figs. 2.12, 2.13A, 2.13B).

The individual cells of the linings are seen to be broad, flat and smooth-walled (Figs. 2.12, 2.13A, 2.13B). Numerous short processes are seen to remain along the joins between adjacent cells, marking the boundaries between the cells (Figs. 2.12, 2.13A, 2.13B). Besides these short processes, the lining cells tend to have a single longer process which projects into the lumen of the channels (Figs. 2.12, 2.13A, 2.13B). It could be suggested that these longer processes may be cilia, the beating action of which might set up fluid currents in the developing channels. However, the positive identification of these processes was not made in the present study, since no TEM observations were carried out.

As the mesenchymal cells become aligned to form these channel linings, their outer (i.e., non-luminal) surfaces are seen to maintain contact with the blood-islands, the endoderm and the ectoderm (Fig. 2.12). The cells of the channel linings are therefore seen to form cellular linings over these surfaces (Fig. 2.16). Meanwhile the small blind channels are seen to coalesce with each other to form larger and larger channels (Figs. 2.17, top and middle micrographs). These in turn unite to form the large cell-lined spaces of the extraembryonic coelom (Fig. 2.17, bottom micrograph, Fig. 2.18). In agreement with Murphy and Carlson (1978), the dorsal lining of the extraembryonic coelom (the somatopleure) is seen to be a single layer of flattened cells apposed to the ectoderm, while the ventral lining of the extraembryonic coelom (the splanchnopleure) is seen to be a single layer of flattened cells which overlies the blood-islands and the yolk sac endoderm (Fig. 2.17, bottom micrograph, Fig. 2.18).

Up to this stage the blood-islands are seen to be still in direct contact with the underlying endoderm by means of long narrow processes (Figs. 2.15, 2.18). These processes appear to emanate from both the blood-island- and the underlying endodermal cells, and they span the space between them (Figs. 2.15, 2.18). The presence of cellular processes which extend between the blood-island cells and the underlying

(Text continues on page 47).

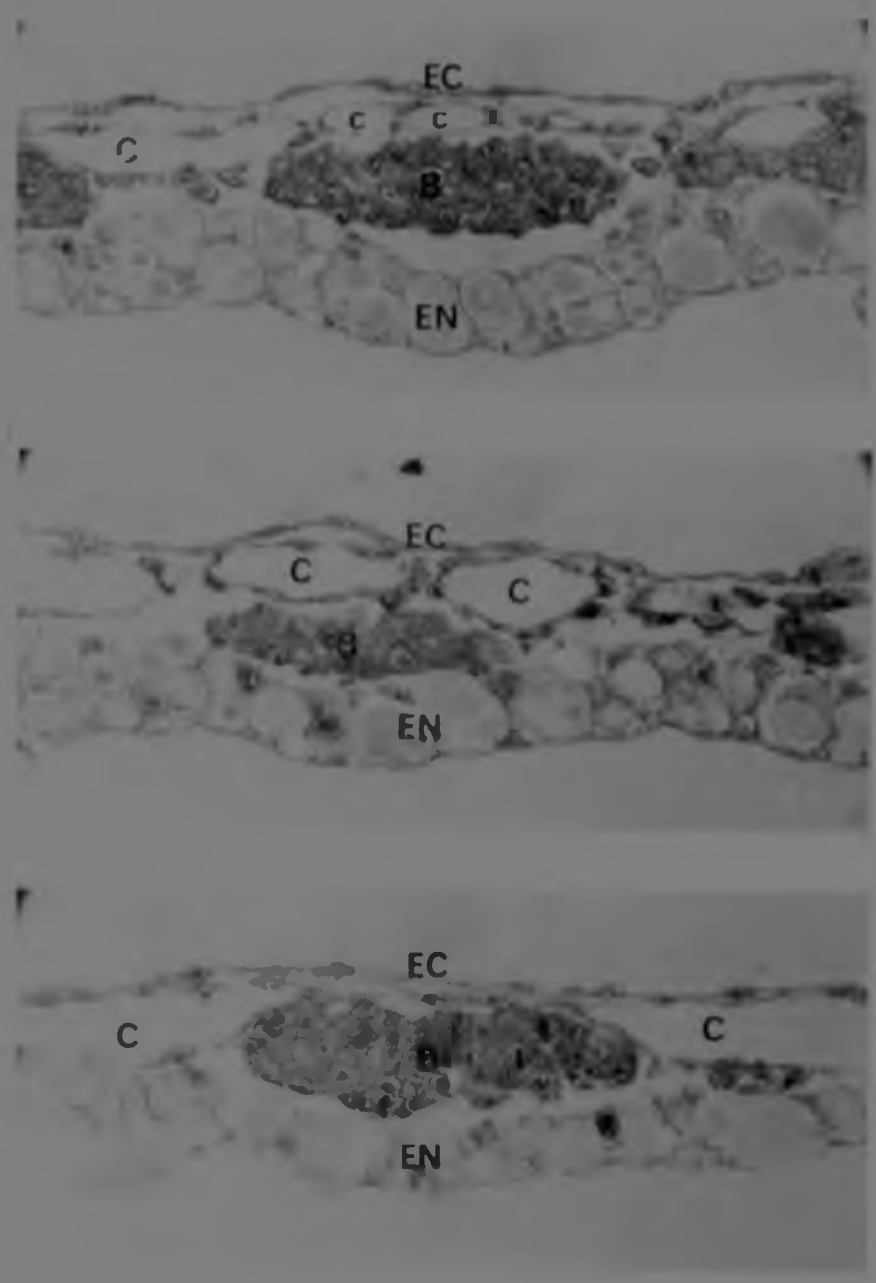


Figure 2.17

Light micrographs of 4-7 μm wax embedded sections through the area opaca vasculosa of a stage 9 chick blastoderm. The mesenchymal cells in the space around the blood-islands (B) are seen to have become aligned to form the wall of small blind channels (C) (top micrograph). The small channels are seen to coalesce with each other to form larger channels (C) (middle micrograph). These, in turn, unite to form the large cell-lined spaces of the extra-embryonic coelom (C) (bottom micrograph).

B, blood-island; C, channels; EC, ectoderm; EN, endoderm.

x 417

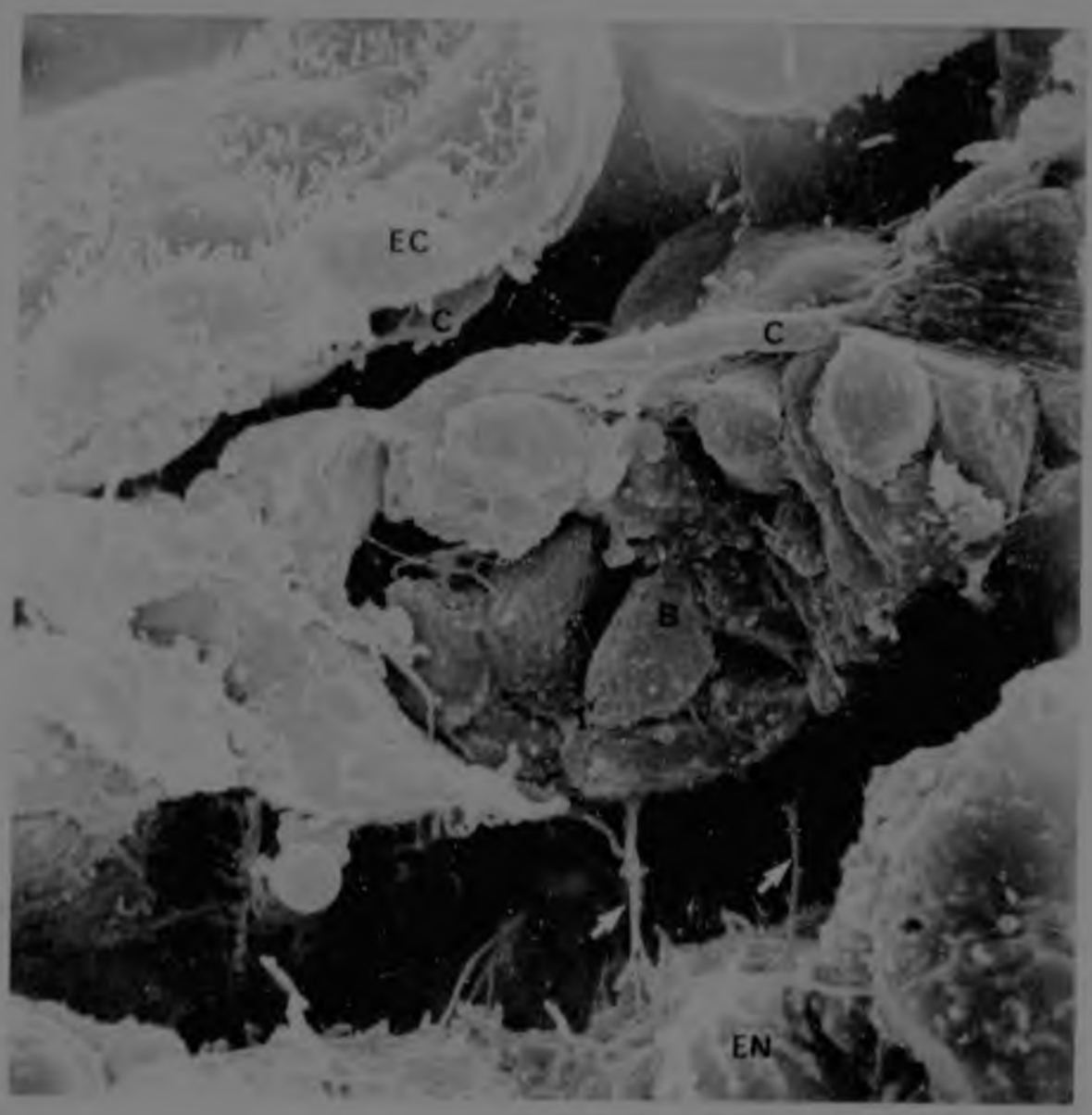


Figure 2.13

Scanning electron micrograph of the area opaca vasculosa of a stage 7 chick blastoderm. The coelom-lining cells (C) are seen to form a single-cell-thick lining below the ectoderm (EC) and over the surface of the blood-island (B). Ventrally, the blood-island cells are seen to make contact with the underlying endoderm (EN) by means of long narrow processes (arrows) which span the space between the blood-island and the endoderm.

B, blood-island; C, coelomic lining cells; EC, ectoderm; EN, endoderm; arrows, processes.

x 5200

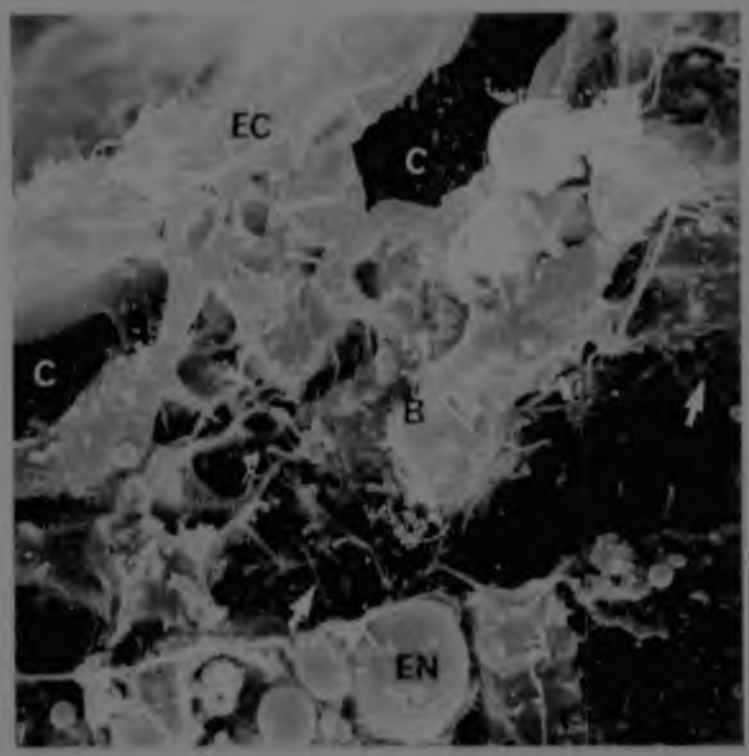


Figure 2.19

Scanning electron micrographs of the area opaca vasculosa of a stage 7 chick blastoderm. Some of the mesenchymal cells (arrows) appear to enter into the space between the blood-islands (B) and the underlying endoderm (EN). These cells will presumably give rise to the ventral splanchnopleuric lining on the ventral surfaces of the blood-islands (see text page 47).

B, blood-island; C, coelom; EC, ectoderm; EN. endoderm; arrows, mesenchymal cells ventral to the blood-islands.

Top x 3976; Bottom x 3880

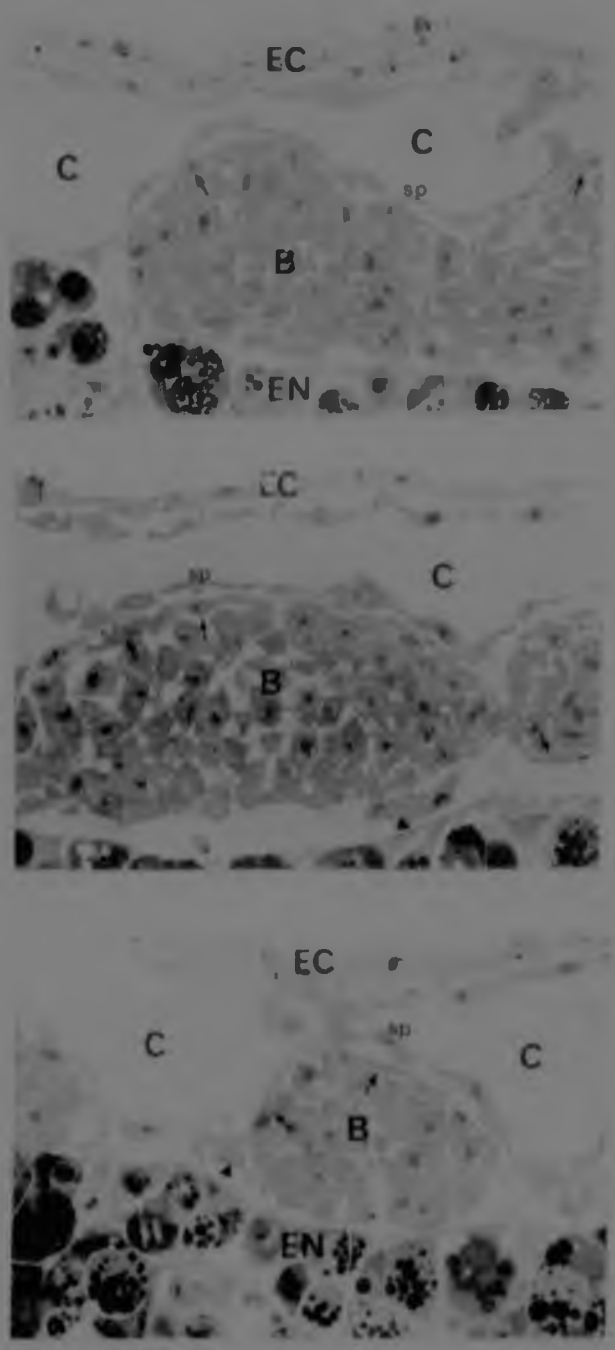


Figure 2.20

Light micrographs of lum plastic embedded sections through the area opaca vasculosa of a stage 8-9 blastoderm. The peripheral cells (arrows) of the blood-islands (B) are seen to have become progressively more elongated and flattened (arrows) and are seen to form a cellular lining over the more central blood-island cells. This layer of flattened cells is the developing vessel endothelium (arrows). The central blood-island cells (B) are thus seen to become surrounded by two adjacent linings, the endothelial lining (arrows) and the splanchnopleure (SP). A number of cells (arrow-heads) are also present in the space between the blood-islands and the underlying endoderm. These cells will presumably give rise to the splanchnopleure ventral to the blood-islands (see text page 47).

B, central blood-island cells; C, coelom; EC, ectoderm; EN, endoderm; SP, splanchnopleure; arrows, developing vessel endothelium; arrow-heads, cells in the space between the blood-islands and endoderm.

x 700

endoderm was also noted by Mato *et al* (1964) in their TEM study.

As the developing splanchnopleuric lining envelops the blood-islands, it appears as if some of these mesodermal cells are also entering into the space between the blood-islands and the underlying endoderm (Figs. 2.19, 2.20). However, the actual entry of these cells is difficult to recognize with certainty in fixed histological material (Figs. 2.19, see also Fig. 2.18), and no previous histological studies have described their actual entry. On the other hand, Fig. 2.20 clearly shows the presence of cells in the space between the blood-islands and the endoderm. Furthermore, the fact that the splanchnopleure does develop ventral to the developing blood vessels was clearly demonstrated in the histological study of Murphy and Carlson (1978), and is also shown in the present study (see Figs. 2.42 to 2.44). It is probable that the cells observed in the space between the blood-islands and endoderm in Fig. 2.20 will form this ventralmost splanchnopleuric lining.

2.3.2.3 The transformation of the peripheral cells of the blood-islands into the endothelial linings

At the same time that the coelom linings are forming, the peripheral cells of the blood-islands are seen to become transformed into the endothelial linings surrounding the more central cells of the blood-islands. The present observations of the cellular events involved in this differentiation are in accordance with the current views in the literature (Mato *et al*, 1964; Edmonds, 1966; Gonzalez-Crussi, 1971; Murphy and Carlson, 1978 - see Section 1.2.2). In agreement with these authors, the endothelial linings are seen to form when the peripheral cells of the blood-islands become progressively more elongated and flattened (Fig. 2.20), forming a cellular lining over the more central cells of the blood-islands (Figs. 2.20, 2.21). As described by Gonzalez-Crussi (1971) and Murphy and Carlson (1978), the blood-islands are thus seen to become surrounded by two adjacent linings, i.e., the endothelial lining and the splanchnopleure (Figs. 2.20, 2.21).

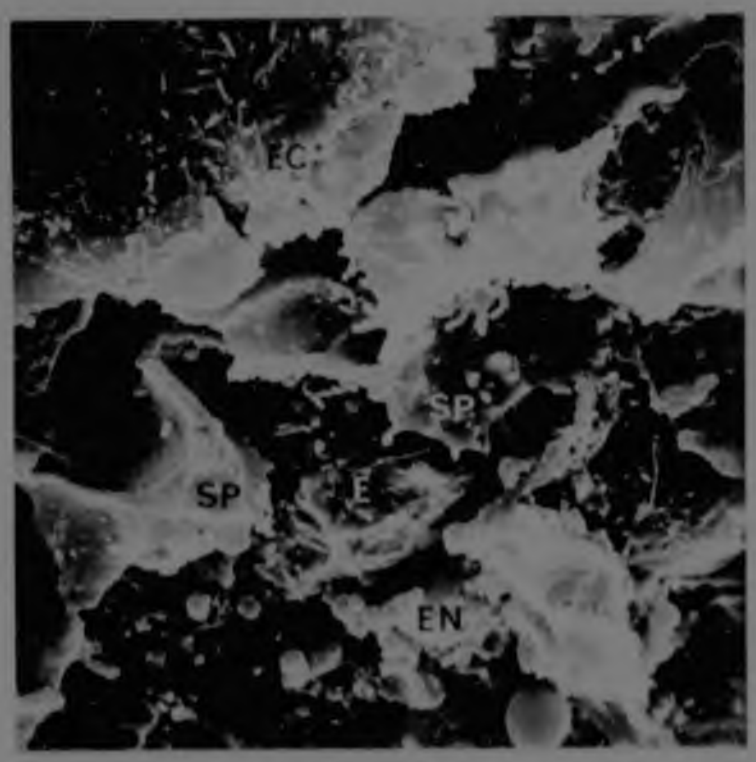


Figure 2.21

Scanning electron micrograph of the area opaca vasculosa of a stage 7-8 blastoderm to show a blood-island (E) which is surrounded by two adjacent linings, the endothelium (E) and the splanchnopleure (SP).

E, endothelium; EC, ectoderm; EN, endoderm; SP, splanchnopleure.

x 4267

2.3.2.4 The joining up of the blood-islands into a plexus

When the blood-islands first form (stage 5-6) they are found to be in the form of discrete clumps of cells (Figs. 2.9, 2.10). However, from stage 7 the individual blood-islands are seen to put out cellular projections (Figs. 2.22, 2.23), described as 'sprouts' by Sabin (1920), by means of which they join up with each other to form a plexus. Successive stages in the joining up of the blood-islands into a plexus can clearly be seen in Fig. 2.24 which shows the AOV of whole-mounted stage 8 and stage 9 blastoderms. This joining up of the individual blood-islands implies some type of cellular recognition and/or attraction between the blood-island cells. As the blood-islands link up and develop their encircling endothelial linings, a network of blood channels containing intravascular blood-

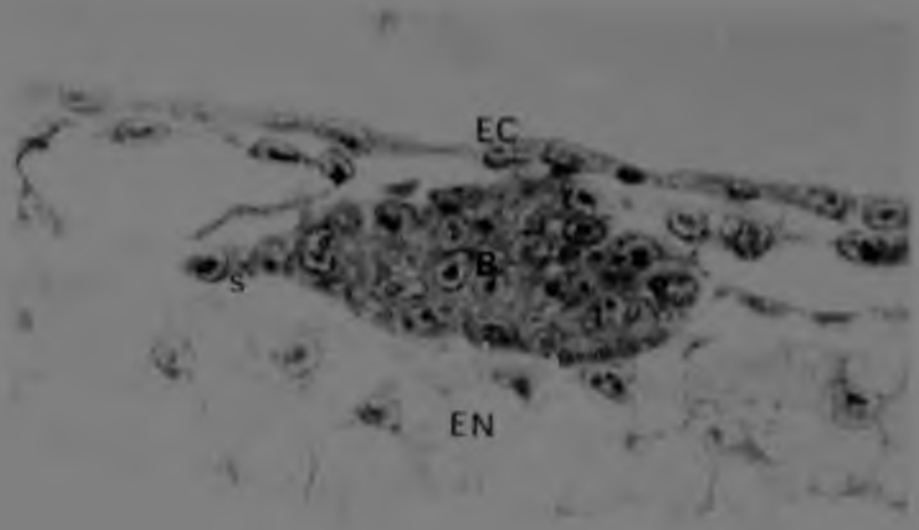


Figure 2.22

Light micrograph of a 4-7µm wax section through the area opaca vasculosa of a stage 7 blastoderm. The blood-island (B) is seen to put out a cellular projection or 'sprout' (S).

B, blood-island; EC, ectoderm; EN, endoderm; S, 'sprout'.

x 1419

island cells is therefore formed in the AOV (Fig. 2.24, bottom micrograph).

2.3.2.5 Further stages in the morphogenesis of the blood vessels and intravascular blood-island cells

Initially the developing endothelial cells of the blood-islands are seen to surround closely the more central cells of the blood-islands (Figs. 2.20, 2.25). However, as found by previous authors (Edmonds, 1966; Gonzalez-Crussi, 1971), the endothelial cells are subsequently seen to become separated from the more central cells of the blood-islands by the formation of extracellular spaces between them (Fig. 2.26).

During the subsequent stages (stages 9 to 10), these extracellular spaces are seen to become greatly enlarged, with a resultant enlargement in the volume of the vessel lumen (Figs. 2.27 to 2.29). During these stages the intravascular blood-island cells are found to

(Text continues on page 53).

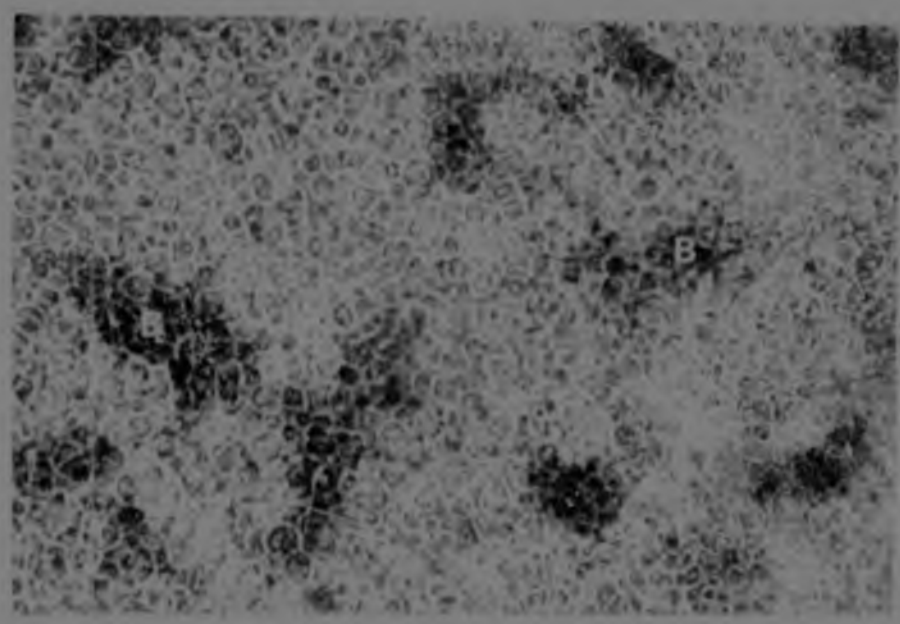


Figure 2.23

Scanning electron micrograph of the area opaca vasculosa of a stage 7-8 chick blastoderm. The blood-island (B) is seen to have put out a cellular projection or 'sprout' (S). Note also that the peripheral cells of the blood-island are seen to be flattened.

B, blood-island; EC, ectoderm; EN, endoderm; S, 'sprout'.

x 7099.



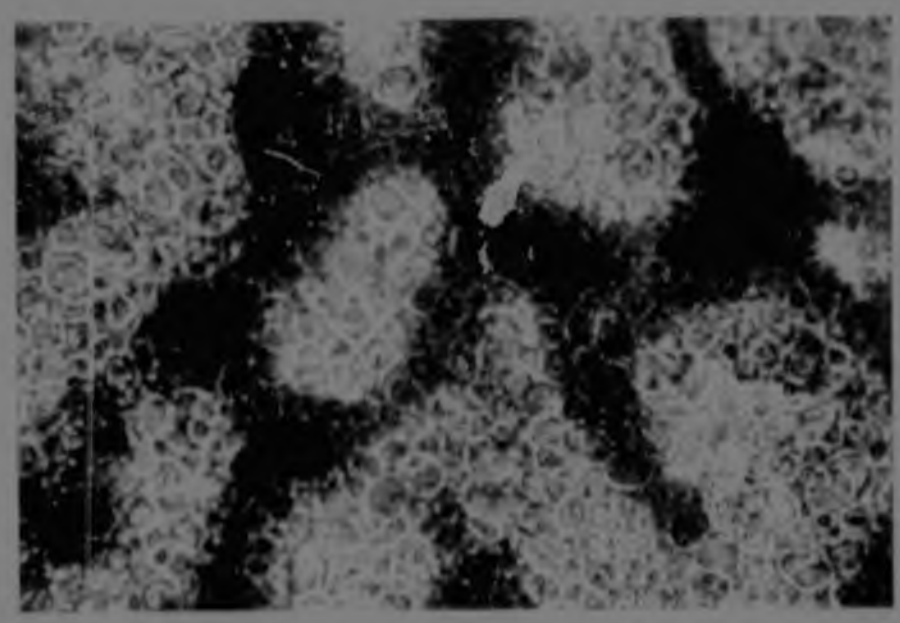


Figure 2.24

Light micrographs of the area opaca vasculosa of whole-mounted stage 8 (top micrograph) and stage 9 (bottom micrograph) chick blastoderms. Successive stages in the joining up of the blood-islands (B) into a plexus can clearly be seen in these two micrographs.

B, blood-islands.

Top x 106; Bottom x 265.



Figure 2.25

Scanning electron micrograph of the area opaca vasculosa of a stage 9 chick blastoderm. The developing endothelial cells (arrow) are seen to surround closely the more central blood-island cells (B).

B, central blood-island cells; EC, ectoderm; EN, endoderm; arrow, endothelial cells.

x 8680

no longer fill the entire vessel lumen (Figs. 2.27 to 2.29). Rather, the major portion of the intravascular blood-islands are seen to remain attached to the dorsal vessel wall and they appear to be carried upwards away from the ventral vessel wall (Figs. 2.27 to 2.29). However, some of the ventral-most cells of these intravascular blood-islands are seen to maintain their attachment to the ventral endothelial wall so that the intravascular blood-islands span the vessel lumen dorso-ventrally (Fig. 2.29). This situation in which the major portion of the intravascular blood-islands becomes attached to the dorsal vessel wall is also illustrated in the textbook of Romanoff (1960) after the 1906 drawing of Rückert and Mollier (see Fig. 1.5B, page 7 of this thesis).

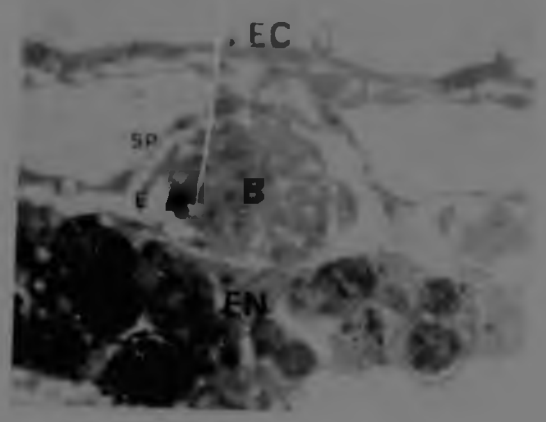


Figure 2.26

Light micrograph of the area opaca vasculosa of a stage 8-9 blastoderm. The developing endothelial cells (E) are seen to have become separated from the more central cells of the blood-island (B) by the formation of extracellular spaces between them (arrow).

B, central cells of blood-island; E, endothelial cells; EC, ectoderm; EN, endoderm; SP, splanchnopleure; arrow, extracellular spaces.

x 416

During these stages the attached ventral-most part of the blood-islands tend to become tapered with the result that the intravascular blood-islands may appear V-shaped (Figs. 2.27, 2.28). In many cases the ventral-most cells in the tapering regions of the intravascular

blood-islands are seen to have become flattened and attenuated (Fig. 2.28). In some instances this attenuation of the ventral-most blood-island cells is so marked that they are seen to form long narrow cellular ribbons which span the vessel lumen dorso-ventrally (Fig. 2.29). The attenuation and flattening of these intravascular blood-island cells suggests that they are being stretched dorso-ventrally across the vessel lumen. This probably occurs as a result of the increase in the diameter of the vessel lumen.

During these stages the peripheral cells of the intravascular blood-islands appear to have become flattened and very closely applied to the more central cells of the intravascular blood-islands (Figs. 2.30 to 2.32). Indeed, the peripheral cells of the intravascular blood-islands appear to form cellular linings over the more central cells of the intravascular blood-islands (Figs. 2.30 to 2.32). This type of observation of the flattening of the peripheral cells of the intravascular blood-islands was originally made in the light microscopic study of Murray (1932), who noted that: "Near the edges of the blood-islands such cells often formed a pseudo-epithelium; that it was not a true epithelium was shown by the fact that the cells were separated from one another, not forming a continuous sheet." (Murray, 1932 pages 504-505).

These observations of the tendency of the peripheral cells of the intravascular blood-islands to become flattened and attenuated, and to form cellular linings, are significant because it occurs when the blood-island cells are already intravascular. This indicates that the cells of the blood-islands still possess the ability to become flattened and to form surface linings even when they are already surrounded by an endothelium.

2.3.2.6 The dissolution of the intravascular blood-island cells

The tendency of the cells of the intravascular blood-islands to be flattened and attenuated appears to be a transient feature in their morphogenesis. From stage 10 to 11 the cells of the intravascular blood-islands are gradually seen to become progressively more rounded (Figs. 2.33 to 2.36) and, as found by previous authors

(Text continues on page 62).



Figure 2.27

Scanning electron micrograph of the area opaca vasculosa of a stage 10-11 chick blastoderm. With the enlargement in the volume of the intravascular spaces, the intravascular blood-island cells (B) no longer fill the entire vessel lumen (L). Rather, the bulk of the intravascular blood-island is seen to be attached to the dorsal endothelial wall, and appears to have been carried upwards away from the ventral endothelial wall. However, some of the ventral-most blood-island cells are seen to remain attached to the ventral endothelial wall so that the blood-island spans the vessel lumen dorso-ventrally. The ventral-most part of the blood-island is seen to be tapered so that the intravascular blood-island as a whole appears V-shaped.

B, intravascular blood-island; C, coelom; EC, ectoderm; EN, endoderm; L, vessel lumen.

X 3410



Figure 2.28

Scanning electron micrograph of the area opaca vasculosa of a stage 10-11 chick blastoderm. With the enlargement in the volume of the vessel lumen (L), the bulk of the intravascular blood-island (B) is seen to have been carried upwards away from the ventral endothelial wall (E). However, some of the ventral-most cells of the intravascular blood-island are seen to remain attached to the ventral endothelial wall, and they appear flattened or attentuated (arrows).

B, intravascular blood-island; E, vessel endothelium; EN, endoderm; L, vessel lumen; arrows, flattened or attenuated ventral-most cells of the intravascular blood-island.

x 3565

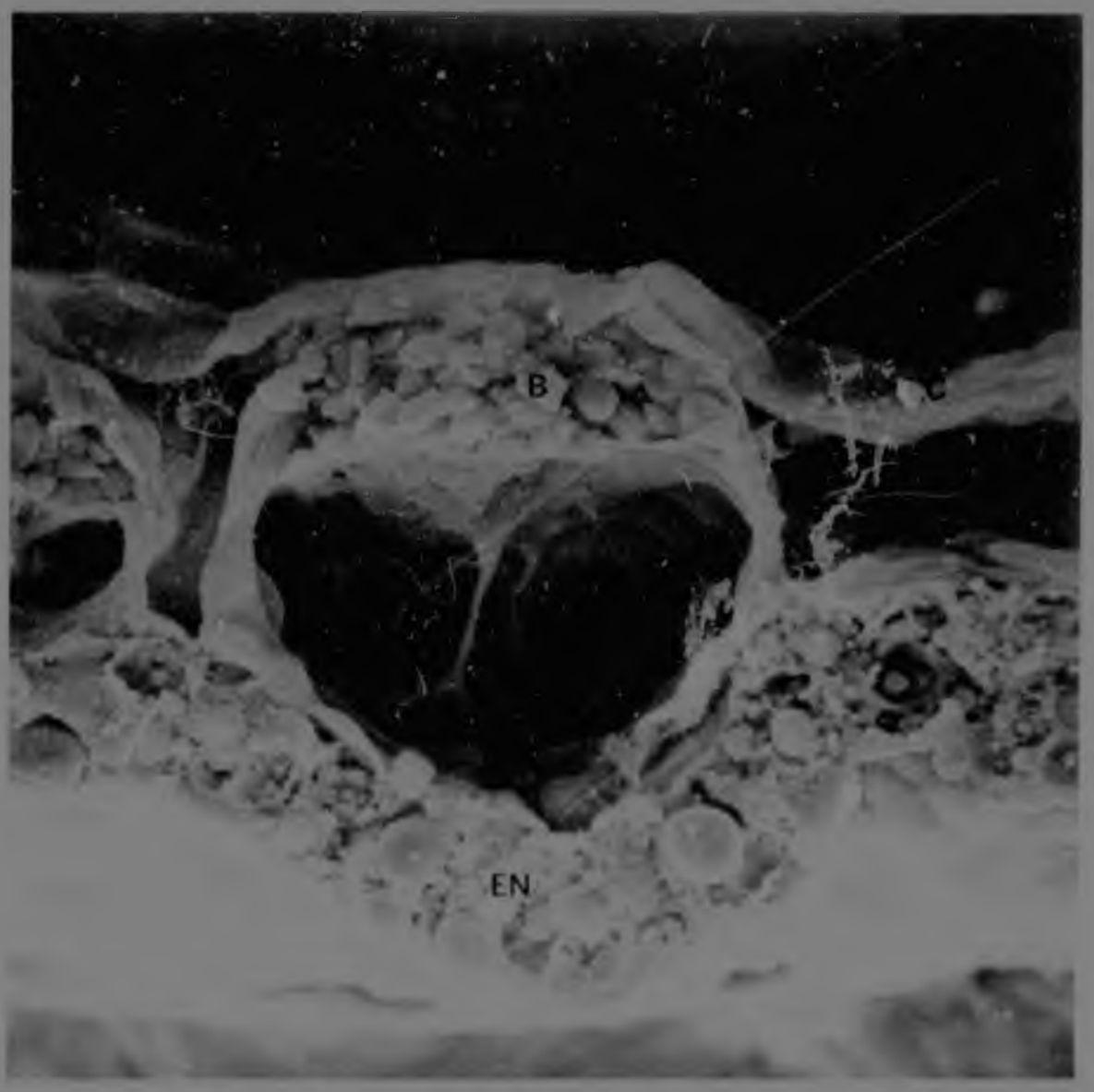


Figure 2.29

Scanning electron micrograph of the area opaca vasculosa of a stage 10-11 blastoderm. The bulk of the intravascular blood-island (B) is seen to be located in the dorsal part of the vessel lumen (L). Some of the ventral-most cells of the intravascular blood-island are seen to have become greatly attenuated (arrow) and they are seen to form narrow ribbons (arrow) which span the vessel lumen (L) dorso-ventrally.

B, intravascular blood-island; C, coelom; EC, ectoderm; EN, endoderm; L, vessel lumen; arrow, attentuated ventral-most cells of the intravascular blood-island.

x 1783



Figure 2.30

Scanning electron micrograph of an intravascular blood-island (B) in the area opaca vasculosa of a stage 10 blastoderm. Some of the peripheral cells of the intravascular blood-island are seen to have become flattened and attenuated (arrows).

B, intravascular blood-island cells; EN, endoderm;
L, vessel lumen; W, ventral vessel wall;
arrows, flattened blood-island cells.

x 8060



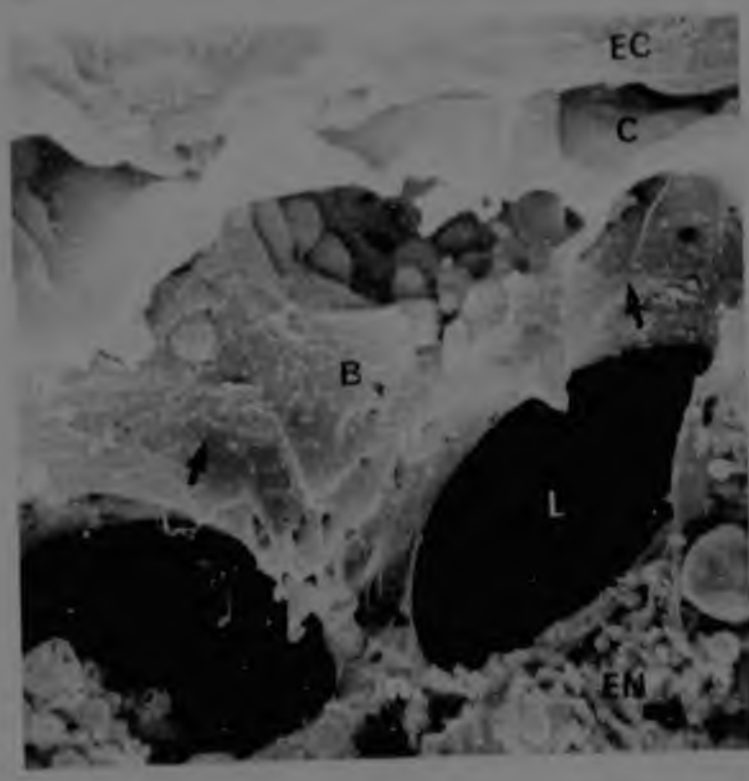


Figure 2.31 (See legend overleaf)

B, cells of intravascular blood-islands; C, coelom; EC, ectoderm; EN, endoderm; L, vessel lumen; arrow, flattened peripheral cells of the intravascular blood-islands; arrow-heads, flattened and attenuated ventral-most cells of the intravascular blood-islands.

x 2425

Figure 2.31 (See photographs on previous page)

Scanning electron micrographs to show the appearance of intravascular blood-islands (B) in the area opaca vasculosa of stage 11 blastoderms. The major portions of the intravascular blood-islands are situated in the dorsal part of the vessel lumens (L). The peripheral cells of the intravascular blood-islands tend to be flattened (arrows) and they tend to form flattened linings over the surfaces of the blood-islands. Likewise, the ventral-most cells of the intravascular blood-islands are seen to be flattened and attenuated (arrow-heads) and they stretch dorso-ventrally across the vessel lumens (L).

B, cells of intravascular blood-islands; C, coelom; EC, ectoderm; EN, endoderm, L, vessel lumen; arrow, flattened peripheral cells of the intravascular blood-islands; arrow-head, flattened and attenuated ventral-most cells of the intravascular blood-islands.

x 2425

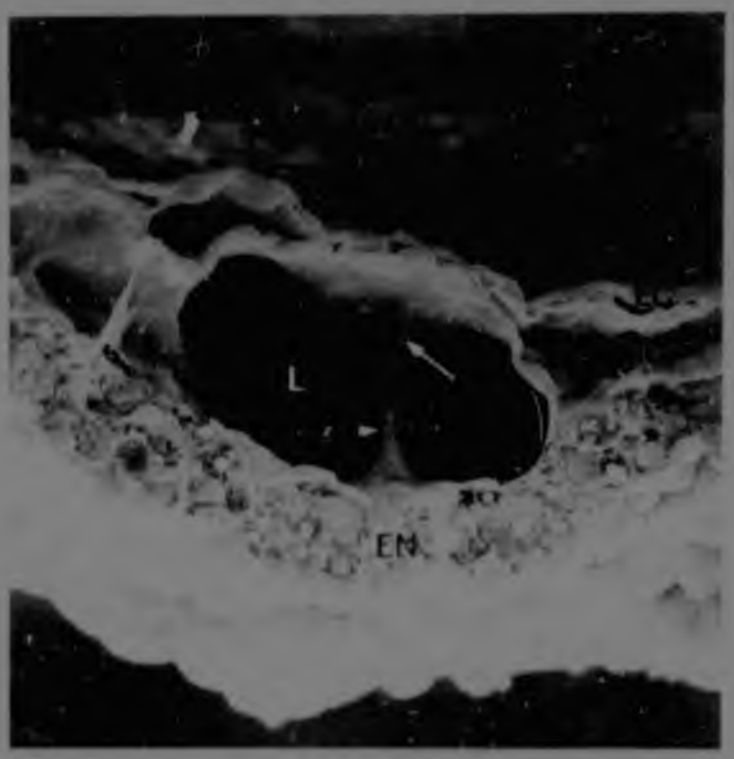


Figure 2.32

Scanning electron micrograph of a blood vessel (L) containing an intravascular blood-island (B) in the area opaca vasculosa of a stage 10-11 blastoderm. The major portion of the intravascular blood-island (B) is situated in the dorsal part of the vessel lumen (L). The peripheral cells of the intravascular blood-island are seen to be flattened (arrows), forming a cellular lining over the more central cells of the intravascular blood-island. The ventral-most cells of the intravascular blood-island are likewise flattened and attenuated (arrow-head), and they stretch dorso-ventrally across the vessel lumen (L).

B, intravascular blood-island; C, coelom; EC, ectoderm; EN, endoderm; L, vessel lumen; arrows, flattened peripheral cells of the intravascular blood-island; arrow-head, attenuated ventral-most cells of the intravascular blood-island.

x 1153.

(Murray, 1932; Edmonds, 1966, Gonzalez-Crussi, 1971; Small and Davies, 1972) they begin to become liberated into the vessel lumens as the first developing blood cells (Fig. 2.37). Figures 2.33 to 2.36 show a series of stages during which the cells of the intravascular blood-islands round up and start to become liberated into the vessel lumens. (Compare these figures with Fig. 2.32). The cells of the intravascular blood-islands which were previously flattened (see Fig. 2.32) therefore lose their flattened morphology and likewise become more rounded (Figs. 2.33 to 2.36). (This situation contrasts with that of the endothelial cells which are blood-island cells which retain their flattened morphology). The rounding off of the intravascular blood-island cells tends to occur first in the dorsal portions of the blood-islands (Figs. 2.33, 2.35) and later starts to occur in the ventral-most pillars of the blood-islands as well (Fig. 2.36). In this manner the cells of the intravascular blood-islands become liberated into the vessel lumens as the first developing blood cells (Murray, 1932; Edmonds, 1966; Gonzalez-Crussi, 1971).

2.3.2.7 Further observations of the yolk sac blood vessels

Also observable in the lumens of the blood vessels during these stages (stages 10 to 12) are large dorso-ventral cellular 'columns' (Figs. 2.34, 2.37, 2.38). In cross section these cellular columns are seen to be hollow, composed of two flattened linings separated by an intercellular space (Figs. 2.37, 2.38). It appears that these cellular columns may have arisen from the juxtaposition of two adjacent blood vessels in the plexus (Fig. 2.37). It is suggested that with the coalescence of adjacent vessels in the plexus, small areas of the original apposed vessel linings remained intact (Figs. 2.37, 2.38), thus forming these dorso-ventral columns (see diagram below). It should be noted that these cellular columns are distinct from the dorso-ventral blood-island pillars described above, although they are similar in appearance in the SEM (Figs. 2.33, 2.34, 2.35). These cellular columns are often seen to remain intact within the vessel lumen even after the complete dissolution of the intravascular blood-islands and their blood-island pillars (Fig. 2.39). Presumably these cellular columns would finally become disrupted when the flow

(Text continues on page 68).

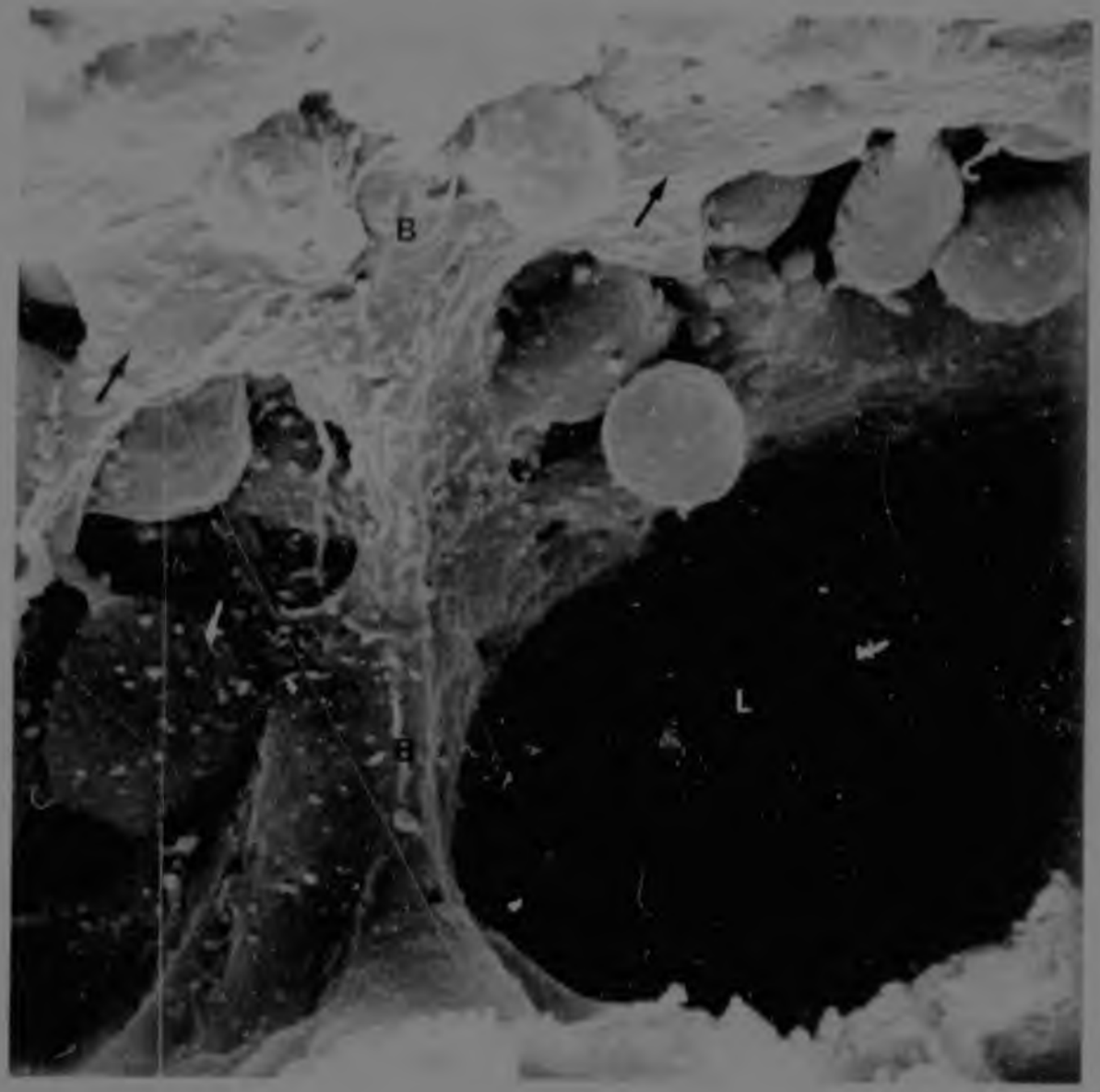


Figure 2.3

Scanning electron micrograph of an intravascular blood-island (B within the lumen (L) of a blood vessel in the area opaca vasculosa of a stage 11 blastoderm. Some of the peripheral cells of the blood-island are seen to be flattened (arrows) and they tend to form a cellular lining over the surface of the blood-island. Likewise, the ventral-most blood-island cells appear flattened and attenuated (arrow-heads) and they tend to form a pillar of cells which stretches dorso-ventrally across the vessel lumen (L). Note that some of the blood-island cells are seen to be less flattened, and they appear as discrete, more rounded cells.

B, intravascular blood-island; L, vessel lumen; arrows, flattened peripheral cells of blood-island; arrow-heads, pillar of ventral-most blood-island cells.

x 7750

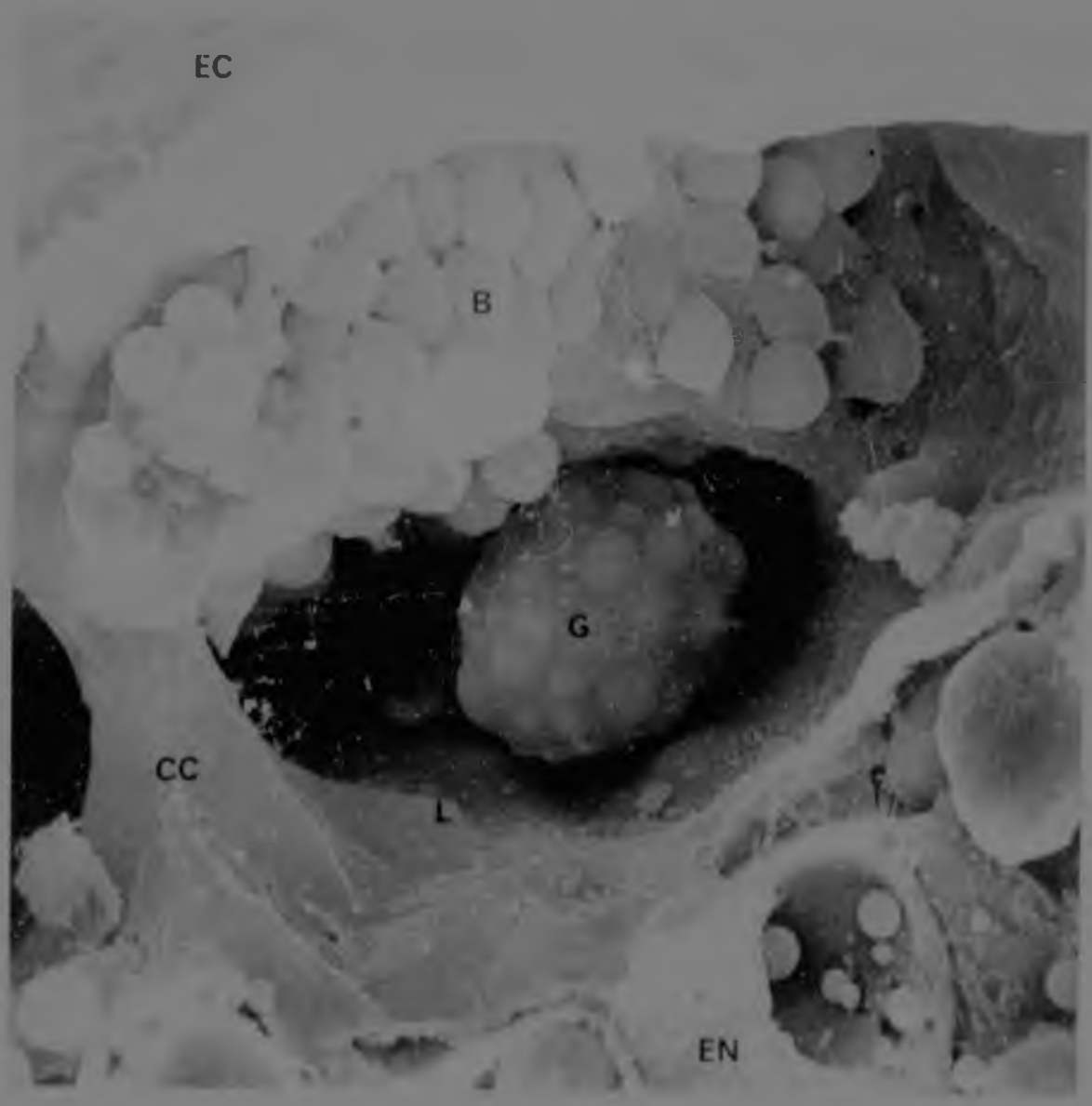


Figure 2.34

Scanning electron micrograph of a blood vessel in the area opaca vasculosa of a stage 10-11 blastoderm. The cells of the intravascular blood-island (B) appear as discrete, more rounded cells. These cells will later become free in the lumen as the first developing blood cells. On the left of the blood-island is a large dorso-ventral cellular column (CC). Although this cellular column is similar in appearance to the ventral blood-island pillar of Fig. 2.33, these two structures are found to be distinct from each other (see text, page 62). The rounded 'golf ball' body (G) ventral to the blood-island is a primordial germ cell within the yolk sac blood vessel (see Section 2.3.5).

B, intravascular blood-island; CC, cellular column; EC, ectoderm; EN, endoderm; G, primordial germ cell; L, vessel lumen.

x 3410

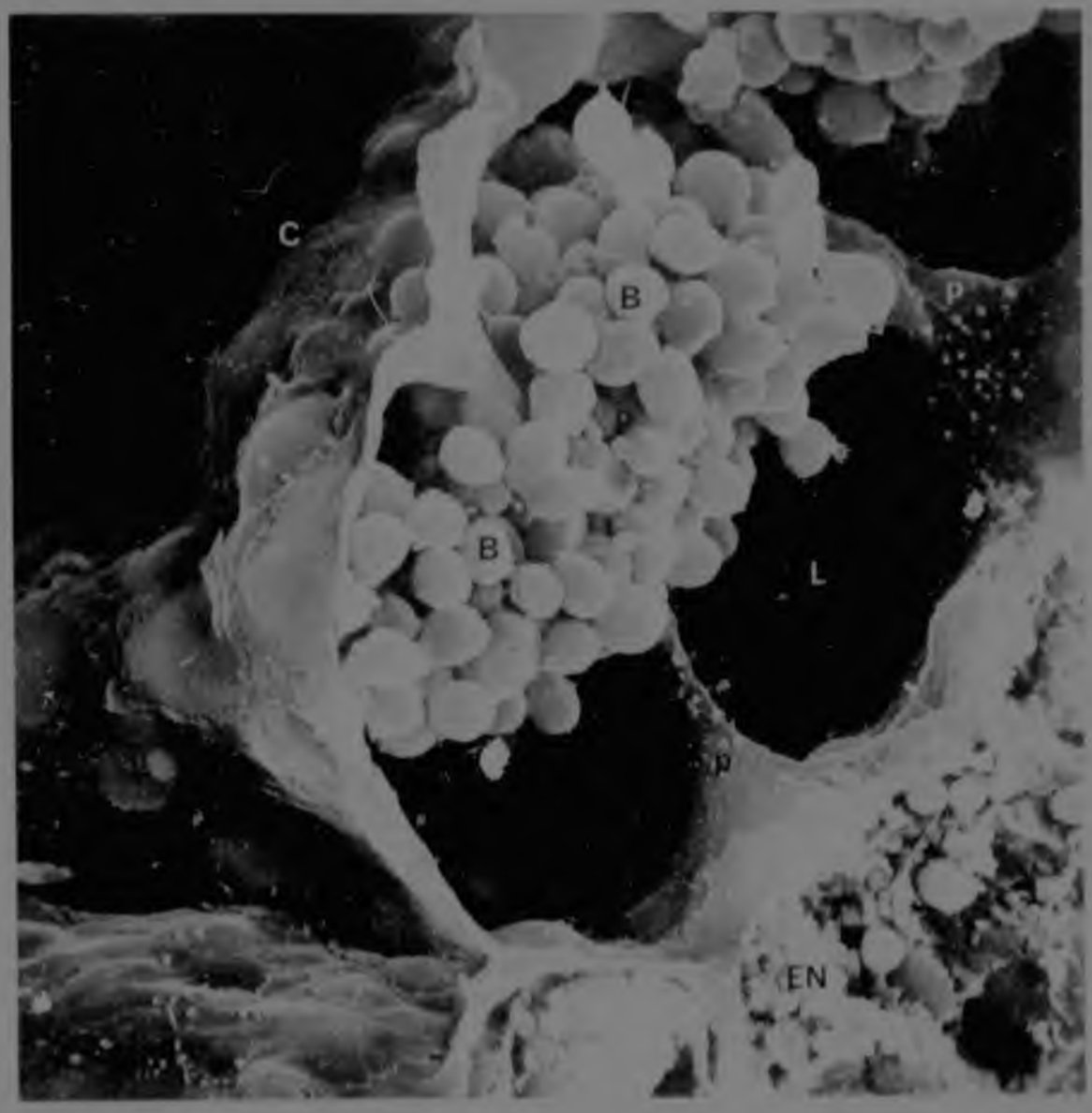


Figure 2.31

Scanning electron micrograph of a blood vessel in the area opaca vasculosa of a stage 10-11 blastoderm. Most of the cells of the blood-island (B) are seen to have become rounded. These cells will soon become liberated into the lumen (L) of the vessel as the first developing blood cells. Also present in the lumen of the vessel are two dorso-ventral cords of attenuated cells (p). The cord on the left appears to pass right through the blood-island, and is therefore probably a cellular column rather than a blood-island pillar. It is difficult to tell for certainty whether the cord on the right is a cellular column or a blood-island pillar (cf. Figs. 2.33 and 2.34, and see text page 62).

B, intravascular blood-island; C, coelom; EN, endoderm; L, vessel lumen; p, dorso-ventral cords.

× 1100

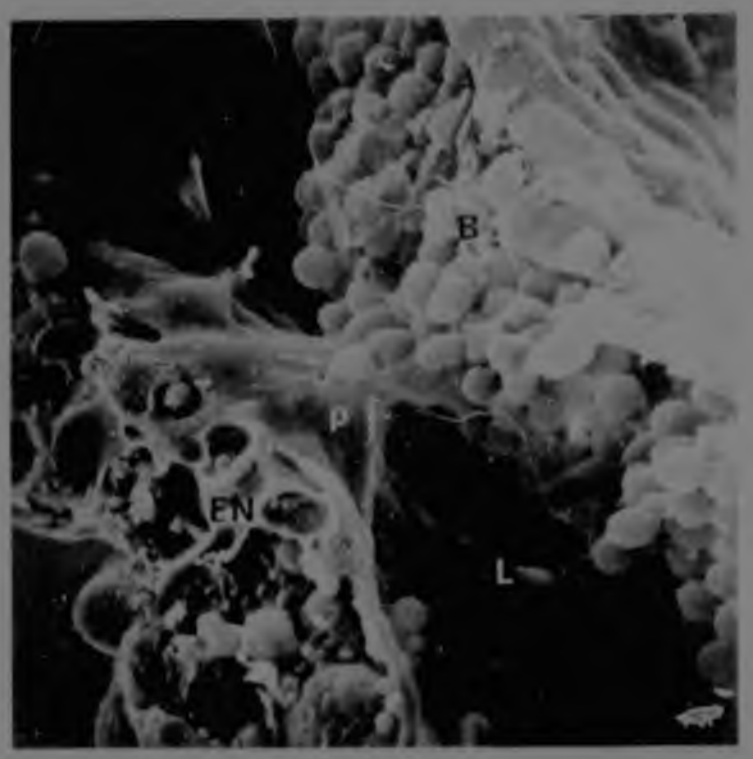




Figure 2.36 (See legend overleaf)

B, intravascular blood-island; EN, endoderm; L, vessel lumen; p, blood-island 'pillar'; arrows, rounded 'pillar' cells.

Top x 1940; Bottom x 5044

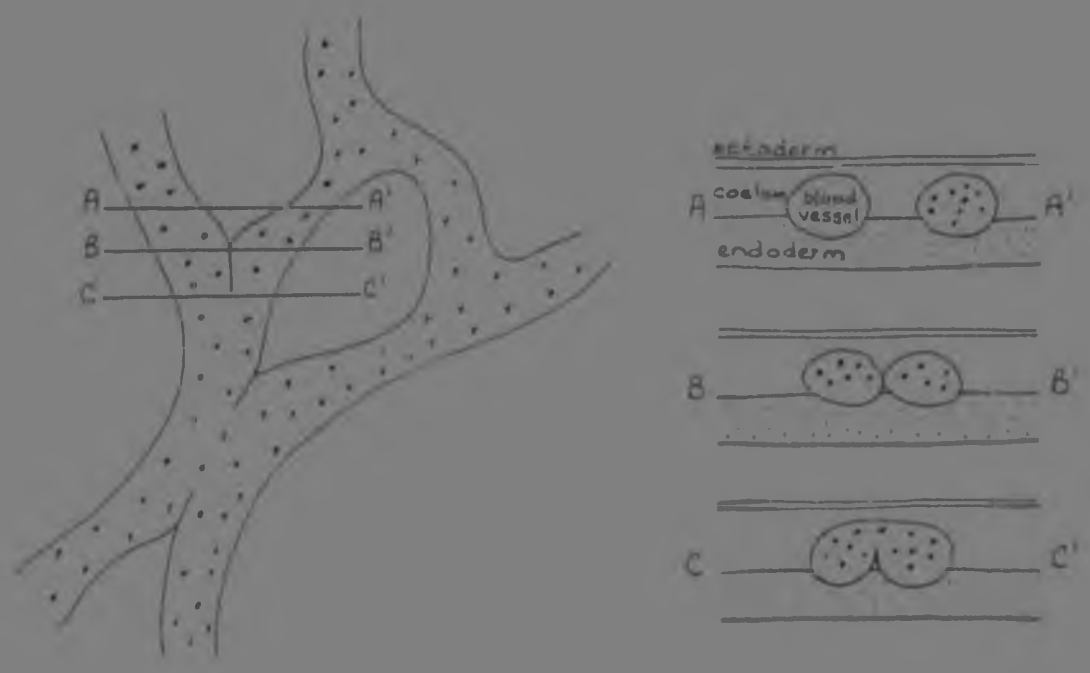
Figure 2.36 (See photographs on previous page)

Scanning electron micrographs of intravascular blood-islands (B) in the area opaca vasculosa of stage 10-11 blastoderms. As the cells of the intravascular blood-islands (B) round off to become liberated into the vessel lumen (L), the ventral-most cells of the intravascular blood-islands, which previously formed the blood-island 'pillars' (p), are likewise seen to become more rounded (arrows). These rounded 'pillar' cells will likewise become liberated into the vessel lumen (L) as the first developing blood cells.

B, intravascular blood-island; EN, endoderm; L, vessel lumen; p, blood-island 'pillar'; arrows, rounded 'pillar' cells.

Top x 1940; Bottom x 5044

of blood through the vessels became stronger.



Surface view (left) of the plexus of blood vessels, and cross sections (right) of the blood vessels at levels A - A^1, B - B^1, and C - C^1.

An examination of the vessel endothelium during these stages (stages 10 to 12) reveals holes in the ventral vessel wall where it overlies the yolk sac endoderm (Figs. 2.41, 2.43). These holes range in size from 0,2µm to 1,3µm in diameter, and are thus smaller in diameter than the 3µm to 4µm holes described by Murphy and Carlson (1978). These holes may be involved in the translocation of materials from the yolk sac endoderm into the yolk sac blood vessels (see Patten, 1971).

In agreement with Gonzalez-Crussi (1971) the yolk sac blood vessels during these stages appear large and sinusoidal (Figs. 2.39, 2.41, 2.42). As described in the TEM study of Gonzalez-Crussi (1971) and in the TEM and SEM study of Murphy and Carlson (1978), the walls of the vessels are seen to be bilayered, i.e., composed of an endothelial lining which is closely surrounded by the splanchnopleure (Fig. 2.42). It is found in the present study that the splanchnopleure covering the dorsal part of

(Text continues on page 70)

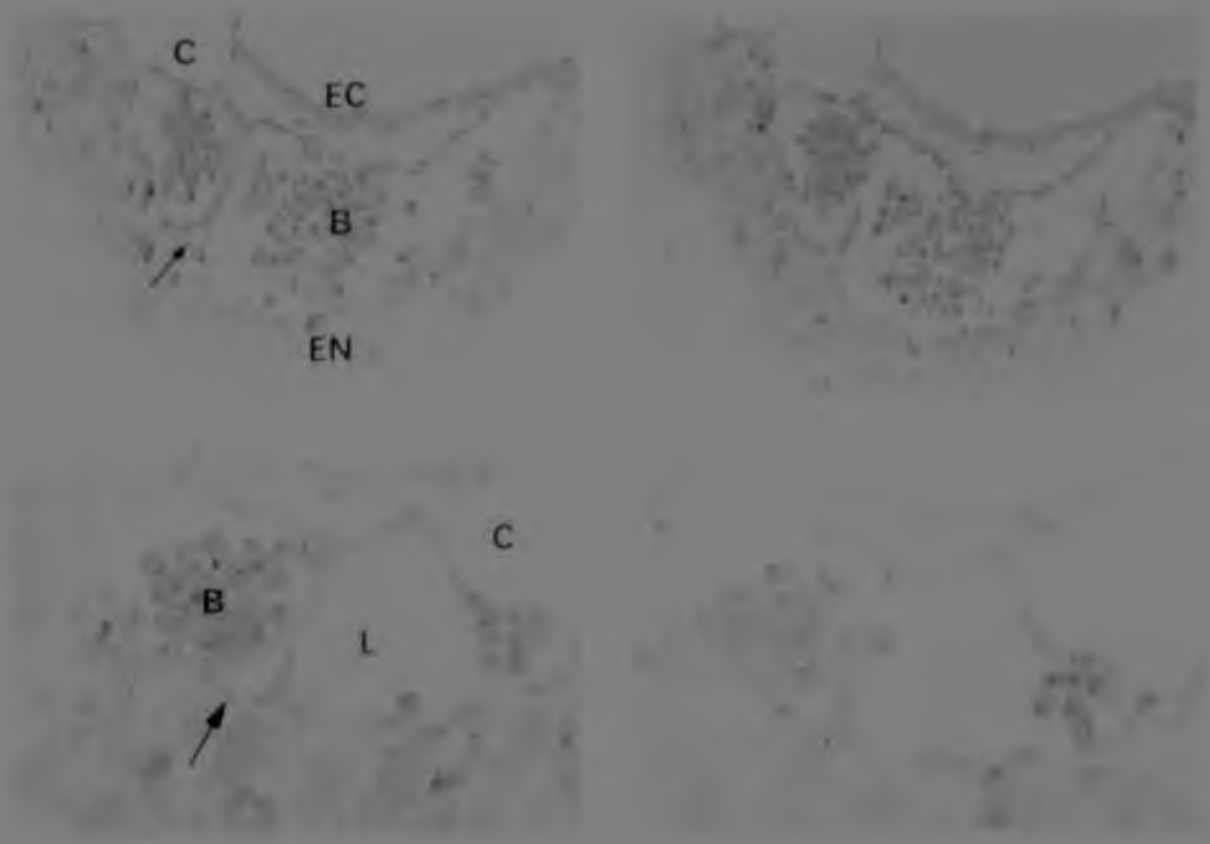


Figure 2.37

Light micrographs of blood vessels in the area opaca vasculosa of stage 11-12 chick blastoderms. Spanning the lumens (L) of the blood vessels are hollow dorso-ventral cellular 'columns' (arrows). The cellular columns are seen to be composed of two flattened cellular linings, separated by an intercellular space. These dorso-ventral columns have probably arisen from the juxtaposition of the walls of adjacent blood vessels in the plexus (see text and diagram, pages 62 and 68, respectively).

B, first developing blood cells; C, coelom; EC, ectoderm; EN, endoderm; L, vessel lumen; arrows, hollow 'columns'.

Top serial sections x 355
Bottom serial sections x 711



Figure 2.38

Scanning electron micrograph of a dorso-ventral cellular 'column' (CC) within the lumen (L) of a yolk sac blood vessel of a stage 11 blastoderm. The column (CC) has been cut open, and its hollow nature is clearly evident. It is seen to be composed of a flattened cellular lining which surrounds an intercellular space.

B, first developing blood cells; CC, hollow 'column';
EN, endoderm; L, vessel lumen.

x 4849

the vessel is so closely applied to the dorsal vessel endothelium that in SEM sections the two layers tend to appear as a single layer (Figs. 2.35, 2.41, 2.43). However, the present lum plastic embedded sections (Fig. 2.42), and the TEM sections of Gonzalez-Crussi (1971) and Murphy and Carlson (1978) confirm the bilayered nature of this region. The splanchnopleure covering the ventral part of the vessel endothelium appears to be less tightly adherent to the ventral endothelium since it is usually clearly observable, even in the SEM (Figs. 2.40, 2.41, 2.43, 2.44).

(Text continues on page 77).

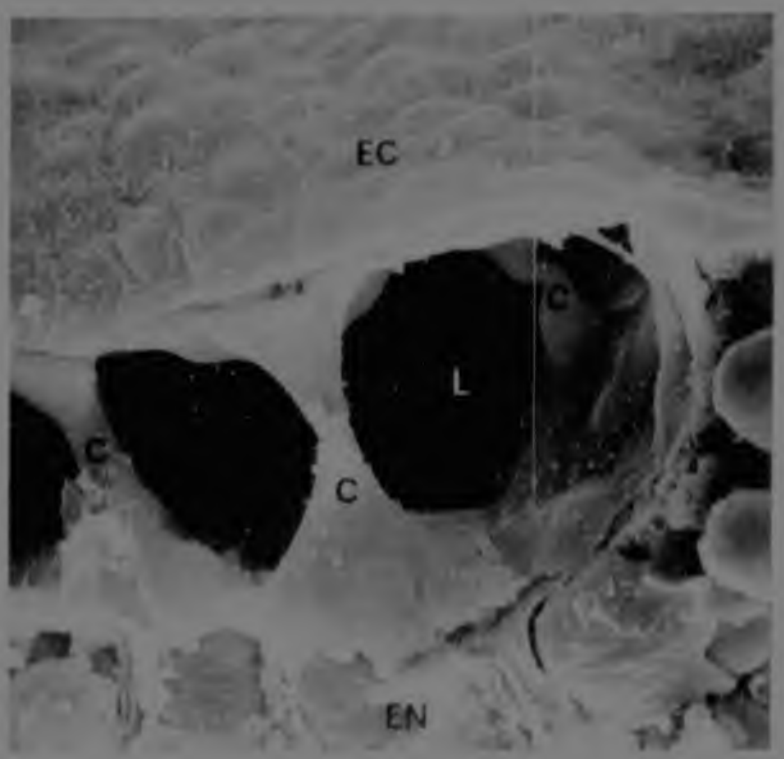


Figure 2.39

Scanning electron micrograph of a blood vessel (the terminal sinus) in the area opaca vasculosa of a stage 11 blastoderm. A number of dorso-ventral cellular columns (C) remain intact within the vessel lumen (L) even after the complete dissolution of the intravascular blood-islands and their blood-island pillars.

C, cellular columns; EC, ectoderm; EN, endoderm;
L, vessel lumen.

x 2135

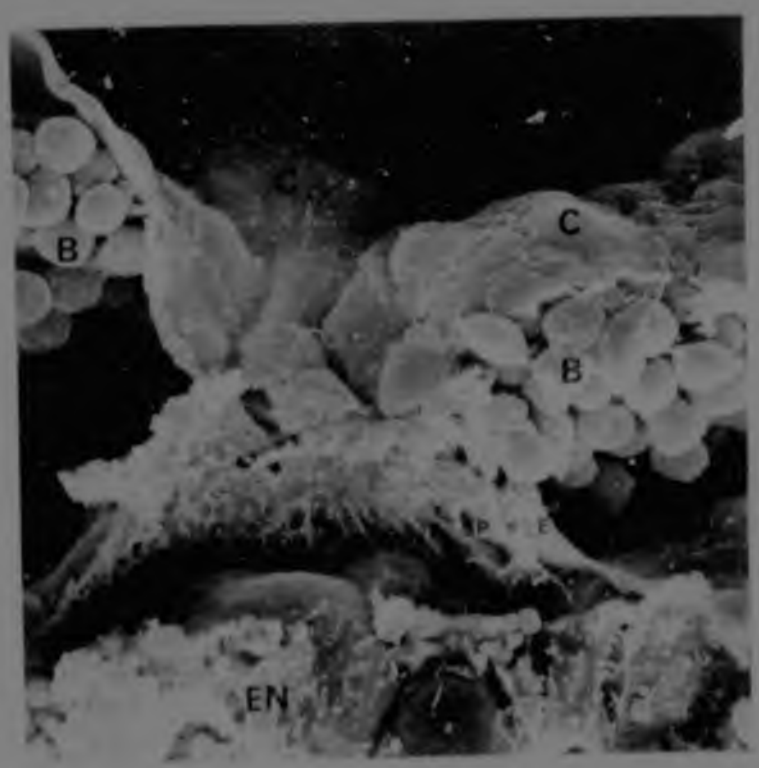


Figure 2.40

Scanning electron micrograph of a blood vessel in the area opaca vasculosa of a stage 11 blastoderm. The splanchnopleure (SP) which underlies the ventral vessel endothelium (E) can be clearly discerned when the ventral vessel wall is peeled away from the yolk sac endoderm (EN).

B, blood cells; C, coelom; E, vessel endothelium; EN, endoderm; SP, ventral-most splanchnopleure.

x 2426



Figure 2.41 (See legend overleaf)

C, coelom; EN, endoderm; h, holes in ventral vessel wall; L, vessel lumen; p, long narrow processes.

Top x 4831; Bottom x 5277

Figure 2.41 (See photographs on previous page)

Scanning electron micrographs of a blood vessel (top micrograph) and the ventral aspect of the ventral vessel wall (bottom micrograph) in the area opaca vasculosa of stage 10-11 blastoderms. Holes (h), 0,2µm to 1,3µm in diameter, are visible in the ventral vessel wall where it overlies the yolk sac endoderm (EN). Long narrow processes (p) extend between the ventral vessel wall and the endoderm. In the top micrograph the sinusoidal nature of the blood vessel is clearly evident.

C, coelom; EN, endoderm; h, holes in ventral vessel wall; L, vessel lumen; p, long narrow processes.

Top x 4831; Bottom x 5277

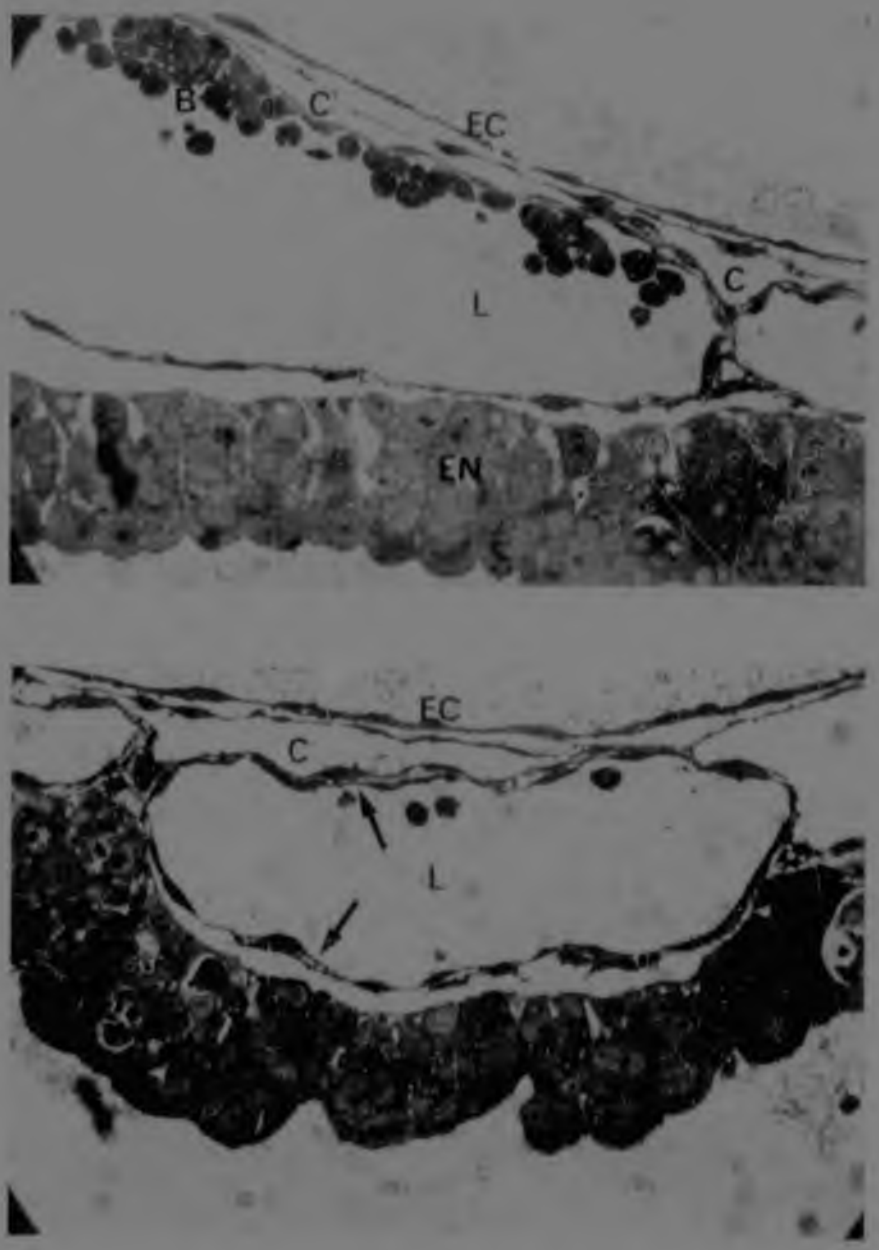


Figure 2.42

Plastic embedded lum sections of blood vessels in the area opaca vasculosa of stage 12 blastoderms. The yolk sac blood vessels (L) appear large and sinusoidal. The walls of the vessels (arrows) are seen to be bilayered, composed of an endothelial lining which is closely surrounded by the splanchnopleure. The somatopleure underlies the ectoderm (EC).

B, developing blood cells; C, coelom; EC, ectoderm; EN, endoderm; L, vessel lumen; arrow, bilayered vessel wall.

X 400

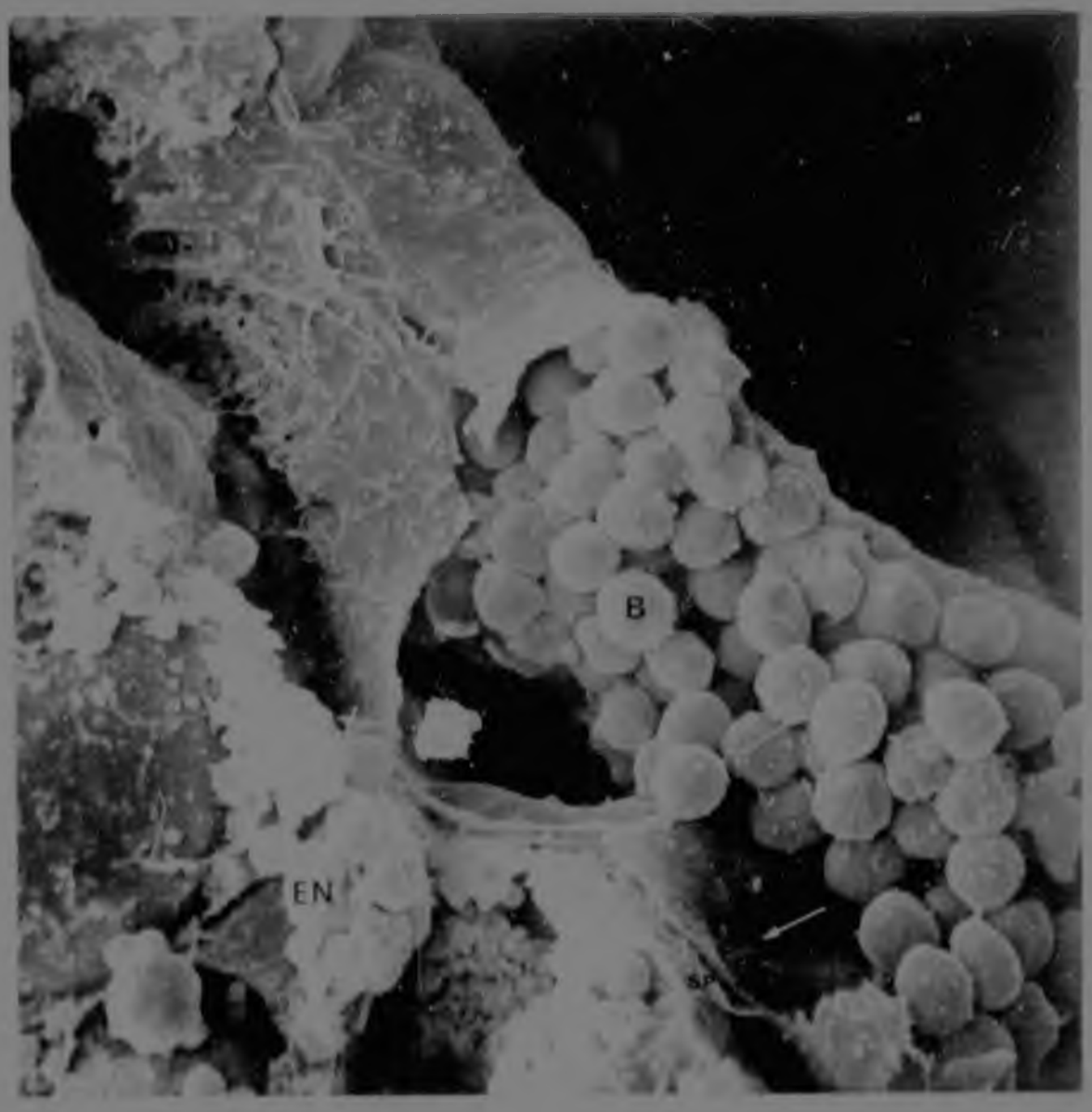


Figure 2.43

Scanning electron micrograph of a blood vessel containing its first developing blood cells (B) in the area opaca vasculosa of a stage 11 blastoderm. A hole (arrow), 0.5 µm in diameter, is present in the ventral vessel wall where it overlies the yolk sac endoderm (EN). The splanchnopleuric lining (SP) ventral to the ventral vessel endothelium (E) can also be discerned.

B, developing blood cells; E, ventral endothelium; EN, endoderm; SP, splanchnopleure; arrow, hole.

x 3875

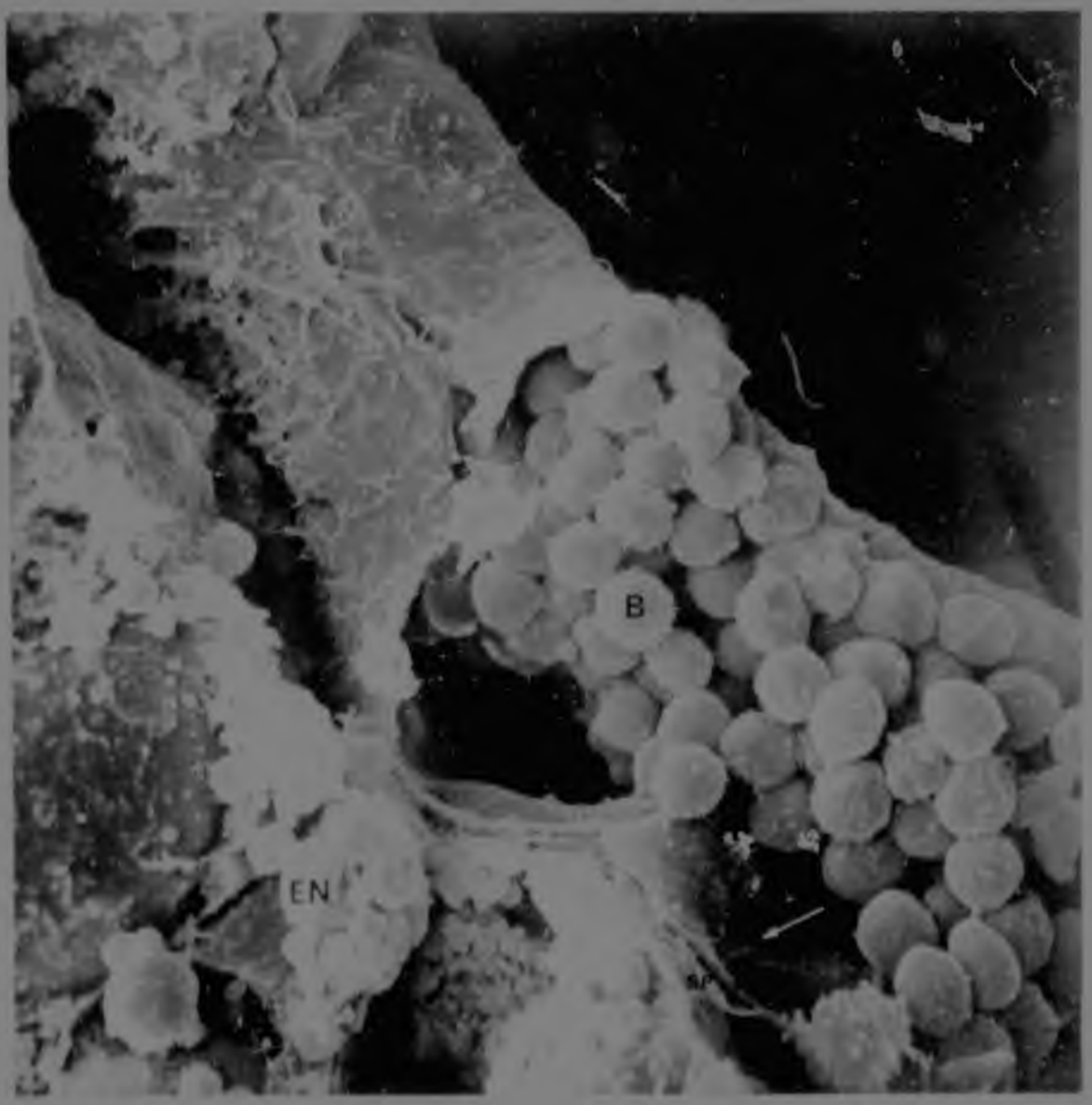


Figure 2.43

Scanning electron micrograph of a blood vessel containing its first developing blood cells (B) in the area opaca vasculosa of a stage 11 blastoderm. A hole (arrow), 0,5 µm in diameter, is present in the ventral vessel wall where it overlies the yolk sac endoderm (EN). The splanchnopleuric lining (SP) ventral to the ventral vessel endothelium (E) can also be discerned.

B, developing blood cells; E, ventral endothelium; EN, endoderm; SP, splanchnopleure; arrow, hole.

x 3875

2.3.2.8 Further observations of the linings of the extra-embryonic coelom

As described above (Section 2.3.2.2), the dorsal lining of the coelom (the somatopleure) forms a flattened cellular layer which underlies the opaca ectoderm, while the ventral lining of the coelom (the splanchnopleure) forms a flattened cellular lining which surrounds the yolk sac blood vessels and covers the yolk sac endoderm (Figs. 2.20, 2.42, 2.44).



Figure 2.44

Scanning electron micrograph of the area opaca vasculosa of a stage 11 blastoderm. The coelom is lined by the somatopleure (S) dorsally and the splanchnopleure (SP) ventrally. Long narrow dorso-ventral cellular processes extend across the coelomic space between the somatopleure (S) and the splanchnopleure (SP). In the present micrograph the splanchnopleure which underlies the ventral vessel endothelium is also discernable (arrow).

E, vessel endothelium; EC, ectoderm; EN, endoderm; L, vessel lumen; S, somatopleure; SP, splanchnopleure; arrow, splanchnopleure underlying ventral endothelium.

x 4075

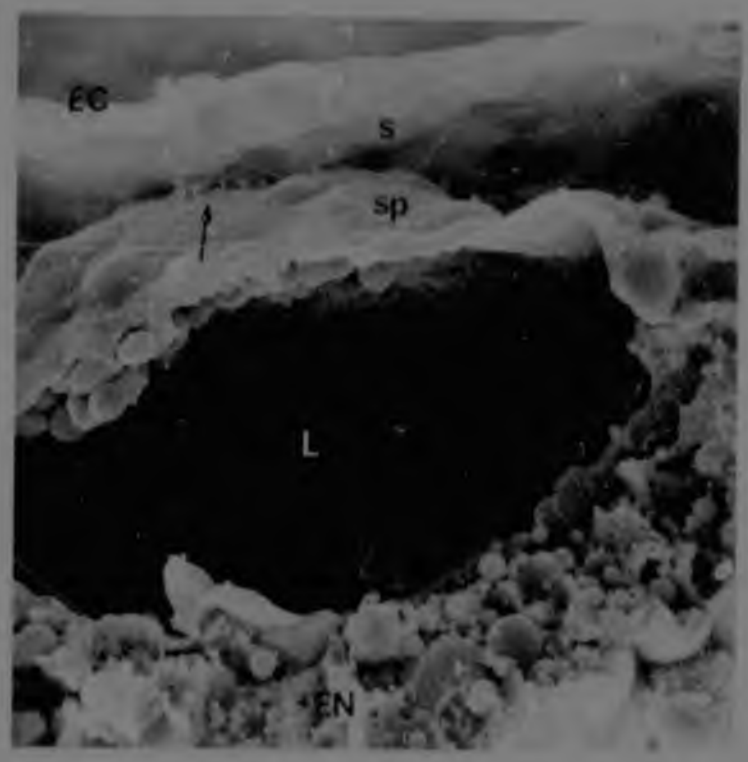


Figure 2.45

Scanning electron micrograph of a blood vessel in the area opaca vasculosa of a stage 10-11 blastoderm. In some regions (see arrow) the somatopleure (S) and splanchnopleure (SP) are seen to be in close physical proximity. In these regions the somatopleure and splanchnopleure are seen to make contact with each other by means of cellular processes (arrow).

B, intravascular blood-island; C, coelom; EC, ectoderm; EN, endoderm; L, vessel lumen; S, somatopleure; SP, splanchnopleure; arrow, cellular processes.

x 1891

In some regions the somatopleure and splanchnopleure are seen to be in close physical proximity (Figs. 2.42, 2.45). Under the SEM it is seen that the somatopleure and splanchnopleure in these regions tend to make contact with each other by means of cellular extensions (Fig. 2.45). In other regions where the somatopleure and splanchnopleure are widely separated from each other, long narrow dorso-ventral cellular processes are often seen to extend between the two layers, across the coelomic space (Fig. 2.44. See also Fig. 2.32). These observations are in contrast to the descriptions of Gonzalez-Crussi (1971) who, from a TEM study, reported

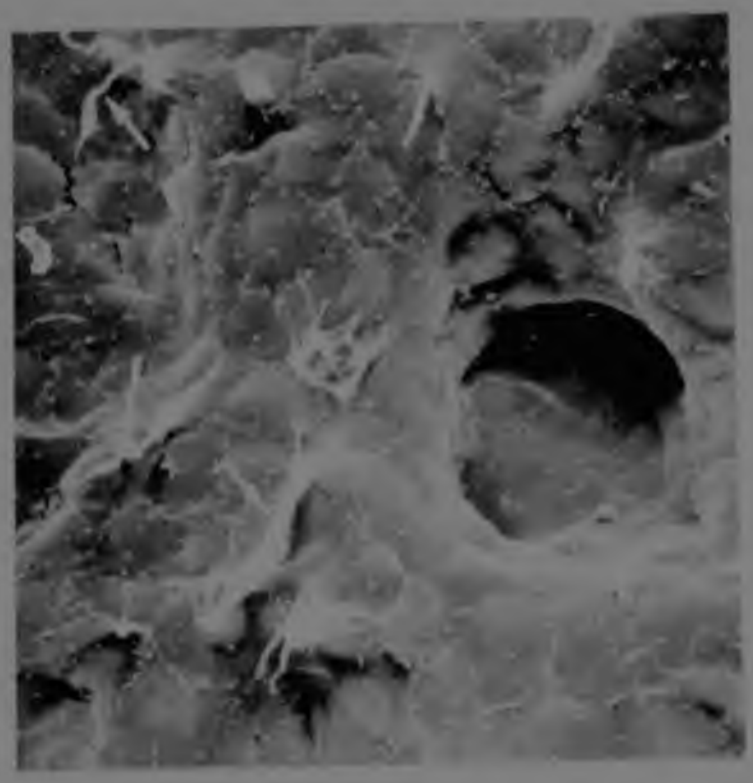


Figure 2.46

Scanning electron micrograph of a surface view of the blood vessels in the area opaca vasculosa of a stage 10 blastoderm to show a large area of the splanchnopleuric lining of the coelom. The ectoderm and the somatopleuric lining of the coelom have been removed. Numerous cellular extensions (arrows) project from the surface of the splanchnopleure across the coelomic space.

arrows, cellular extensions.

x 1940

that no contact was established between the somatopleure and splanchnopleure, even when they were in close physical proximity. However, the present SEM study, in which wide areas of the coelomic linings were observed, reveals the presence of numerous cellular processes between the somatopleure and splanchnopleure (Figs. 2.32, 2.44, 2.45, 2.46). These cellular processes probably arise as a result of the method of formation of the coelom from the coalescence of blind vesicles (see Section 2.3.2.2).

2.3.2.9 General

The above description outlines the cellular interactions and patterning which occur in the AOV during the transformation of an

apparently irregular mass of mesenchymal cells into an ordered system of blood vessels, intravascular blood cells, and coelomic spaces. This transformation is seen to occur in the AOV during stages 5 to 12.

For comparison, the next section (Section 2.3.3) deals with the description of the cellular events involved in the transformation of the mesenchymal cells in the AP into the extraembryonic vascular system and coelomic linings of the AP.

2.3.3 The cellular events involved in vasculogenesis and coelomogenesis in the area pellucida

Whereas the morphogenesis of the yolk sac vascular system first starts occurring in the AOV as early as stages 5 to 6 (see Section 2.3.2 above), the development of this system only starts occurring in the AP at a later stage, from stage 7 to 8 onwards. Indeed, Sabin (1920) found that blood-islands were not constantly observable in the AOV until stage 8+. Thus, whereas at stage 7 to 8 the development of the extraembryonic vascular system and coelom in the AOV is already well under way (see Section 2.3.2), in the AP the mesodermal cells are still mainly mesenchymal in appearance, and are still in the form of an irregular mass of cells (Fig. 2.47).

2.3.3.1 The morphogenesis of the extraembryonic blood vessels in the area pellucida

As found by Sabin (1920), from stages 7 to 8 some of these mesodermal cells in the AP are seen to clump together in juxtaposition to the pellucida endoderm to form small cellular aggregations (Fig. 2.48). These cellular aggregates are the pellucida blood-islands (or angioblasts).

In agreement with the textbook description of Lillie (1919), the pellucida blood-islands (angioblasts) are found to be relatively small compared with the blood-islands which develop in the AOV (compare Fig. 2.48 with Figs. 2.10 and 2.17). Furthermore, as described in Lillie's textbook (1919), it is found that whereas the blood-islands in the AOV give rise to blood vessels containing large numbers of intravascular blood cells (see Section 2.3.2 above), the

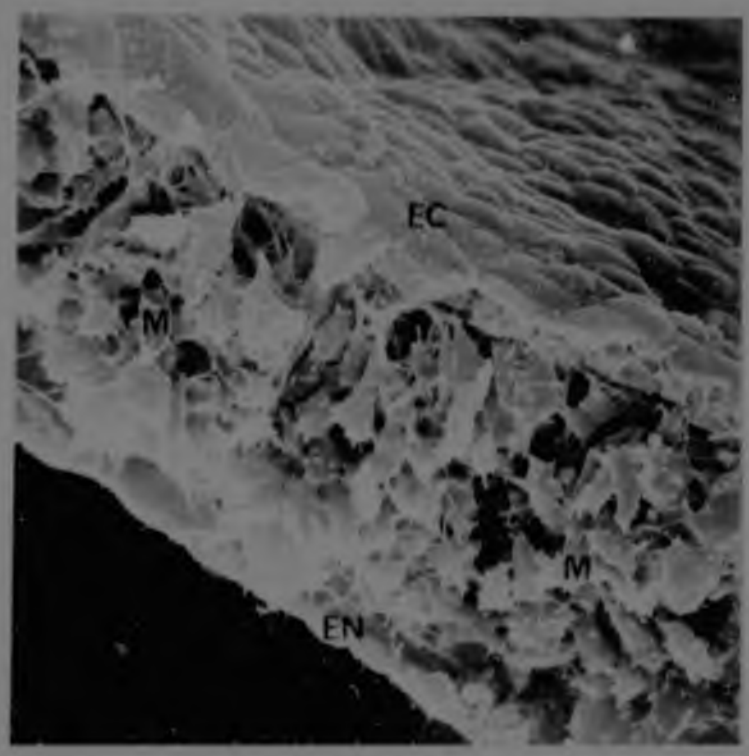


Figure 2.47

Scanning electron micrograph of the area pellucida of a stage 9 blastoderm. The mesodermal cells (M) are still mesenchymal in appearance and they form an irregular sheet of cells in the space between the ectoderm (EC) and endoderm (EN).

EC, ectoderm; EN, endoderm; M, mesenchymal cells.

x 2036

blood-islands (angioblasts) of the AP tend to give rise to empty blood vessels only. This is seen to occur from stage 10 onwards, when extracellular spaces are observed to develop in the centre of the pellucida angioblasts (Figs. 2.49, 2.50). As these spaces enlarge, the cells of the angioblasts are seen to become arranged to form an endothelium surrounding a central lumen (Figs. 2.49, 2.50). These developing blood vessels therefore differ from those that form in the AOV in that they are small and capillary-like (Figs. 2.50, 2.51) rather than large and sinusoidal, and they tend to develop in the absence of the formation of intravascular blood cells.



Figure 2.48

Light micrograph of the area pellucida of a stage 9 blastoderm. Some of the mesodermal cells are seen to have clumped together to form a small cellular aggregation (A) in juxtaposition to the endoderm (EN). These cellular aggregations are the blood-islands (angioblasts) and they are relatively small compared with the size of the blood-islands which develop in the area opaca vasculosa.

A, angioblast; EC, ectoderm; EN, endoderm;
M, mesenchymal cells.

x 400

This description of vessel formation in the AP is similar to the classical description of vessel formation which occurs within the body of the embryo itself (see Balinsky, 1981 page 494). According to this description, "The rudiments of the blood vessels are laid down as aggregations of mesenchyme cells . . . called angioblasts . . . In the amniote body proper . . . the blood vessels develop independently of blood corpuscles. The aggregations of angioblasts become arranged in the form of a flat epithelium surrounding a cavity. The epithelium is the endothelium of the blood vessel . . . " (Balinsky, 1981 page 494).

However, the present description of blood-island differentiation in the AP is in contrast to the descriptions of Sabin (1917, 1920), Sugiyama (1926) and Houser *et al* (1961). As outlined in Section 1.2.3, these authors described the pellucida blood-islands (angioblasts) as developing into blood vessels which subsequently gave rise to intravascular blood cells as well. According to these authors the pellucida angioblasts first became transformed



Figure 2.49

Light micrographs of the area pellucida of a stage 11 blastoderm. Numerous stages in the formation of the pellucida blood vessels are evident. Some of the angioblasts (A) are still solid, whereas in others extracellular spaces (arrows) have formed in the centre of the angioblasts. As these extracellular spaces enlarge, the original angioblasts are seen to become transformed into hollow vessels (L).

A, angioblasts; C, coelom; EC, ectoderm; EN, endoderm;
M, mesodermal cells dorsal to angioblasts;
N, neural tube; L, vessels;
arrows, extracellular spaces in angioblasts.

x 167

into hollow vessels by means of the cellular degeneration (or liquefaction) of the central part of the angioblasts. These authors then maintain that these newly formed pellucida vessels subsequently became filled with intravascular blood-islands which were produced secondarily from the budding off (by mitotic division) of endothelial cells from the vessel walls themselves into the lumens of the vessels.

However, according to the present observations in the AP, and the previous TEM studies in the AOV (Edmonds, 1966; Gonzalez-Crussi, 1971), there is no evidence of cellular degeneration during

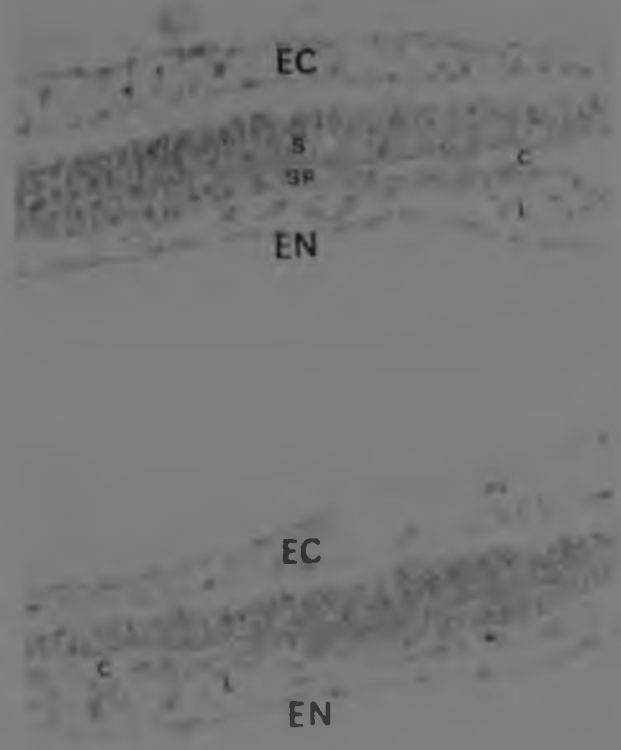


Figure 2.50

Light micrographs of the area pellucida of a stage 11 blastoderm. As the extracellular spaces develop in the centre of the angioblasts the cells of the angioblasts become arranged to surround a central hollow lumen (L). These are the developing pellucida blood vessels. Dorsal to the developing vessels the mesodermal cells are seen to have become aligned to form two layers of cells. The dorsal layer (S) is the fore-runner of the somatopleure, and the ventral layer (SP) is the fore-runner of the splanchnopleure. Clefts (C) develop between these layers and give rise to the coelom.

C, developing coelom; EC, ectoderm; EN, endoderm,
L, developing pellucida blood vessels; S, somatopleure;
SP, splanchnopleure.

x 356

vessel formation (e.g. no pycnotic nuclei are ever observed). Rather, as found in the present study, the angioblastic cells in the AP are seen to become arranged to surround a central cavity (Figs. 2.49, 2.50, 2.55). Furthermore, as observed in the present study, and in agreement with the textbook description of Lillie (1919), the blood vessels which develop in the AP tend to form in the absence of the formation of intravascular blood



Figure 2.51

Light micrograph of the area pellucida of a whole-mounted stage 12 blastoderm. The blood vessels (P) which form in the area pellucida are small and capillary-like. These blood vessels are seen to become secondarily filled with blood cells when the circulation of the blood commences and blood cells are mobilized from the vessels of the area opaca vasculosa.

B, blood vessels; E, body of the embryo.

x 28

cells, there being no evidence for an endothelial origin of blood cells. Rather, it is found that the pellucida blood vessels subsequently become secondarily filled with blood cells when the yolk sac circulation commences, and blood cells are mobilized from the vessels of the AOV into those of the AP. This is likewise in agreement with the early description of Lillie (1919).

In agreement with the description of Lillie (1919) it is found that the cellular events involved in vasculogenesis in the AP grade into those which occur in the AOV, there being an 'intermediate zone' in the border region between the AOV and AP. It is found in the present study that this 'intermediate zone' is particularly obvious in the posterior and posterio-lateral region of the

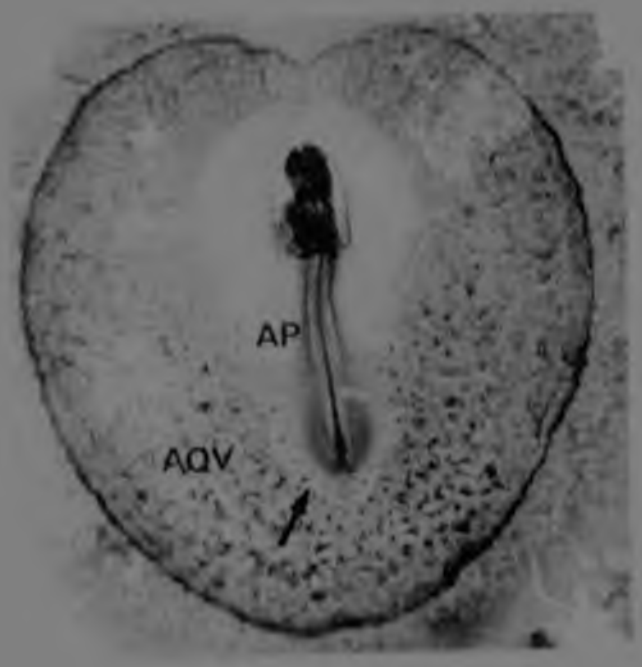


Figure 2.52

Light micrograph of a whole-mounted stage 12 blastoderm. Conditions in the area pellucida (AP) grade into those in the area opaca vasculosa (AOV). There is an 'intermediate zone', particularly evident in the posterior and posteriolateral region of the area pellucida (arrow), in which the blood-islands tend to be larger than those in the rest of the area pellucida. These blood-islands tend to give rise to blood vessels containing small intravascular blood-islands as well.

AOV, area opaca vasculosa; AP, area pellucida; arrow, 'intermediate zone'.

x 5,6

AP (Fig. ' ' As indicated by Lillie (1919), in this region the pell ngioblasts (blood-islands) are found to be larger than those in the rest of the AP (Fig. 2.52), and they tend to give rise to blood vessels containing small numbers of intravascular blood cells as well. Similar observations of the larger size of the posterior pellucida angioblasts (blood-islands) and their direct transformation into both the vessel endothelium and intravascular blood-islands were also recorded by Houser *et al* (1961) and by Sabin (1920) (see Section 1.2.3).

2.3.3.2 The morphogenesis of the extraembryonic coelom in the area pellucida

At the same time that the pellucida blood vessels are forming, the mesodermal cells dorsal to the developing vessels are seen to give rise to the somatopleuric and splanchnopleuric linings of the pellucida extraembryonic coelom (Figs. 2.50, 2.53). As indicated in Section 1.2.4, the present observations of the cellular events involved in coelomogenesis in the AP are in agreement with the descriptions of Sabin (1920). As found by Sabin (1920), the morphogenesis of the pellucida coelom is seen to occur as follows: From stage 7-8 the mesodermal cells dorsal to the developing angioblasts are seen to become aligned to form two adjacent layers of cells (Figs. 2.49, 2.50, 2.53). In agreement with Sabin (1920), the dorsal layer of cells is the fore-runner of the somatopleure and the ventral layer of cells is the fore-runner of the splanchnopleure. Initially, these two cellular layers are closely apposed to each other, and there are no spaces between them (Fig. 2.49, Top micrograph). However, clefts soon appear between the two layers (Figs. 2.49, 2.50, 2.53) with the result that the somatopleure and splanchnopleure become separated from each other (Figs. 2.53, 2.54). The resultant space is the extraembryonic coelom.

These events are different from those which occur during coelom formation in the AOV. As outlined in Section 2.3.2.2, coelomogenesis in the AOV occurs as a result of the arrangement of the mesodermal cells to form the walls of small blind channels. These small channels then coalesce with each other to form larger and larger channels. Finally, the coalescing channels unite to form the large coelomic spaces of the AOV.

Whereas the coelomic linings in the AOV are seen to be simple squamous epithelia (Fig. 2.42), the coelomic linings in the AP vary in nature regionally. Thus, in the medial part of the AP the coelom linings are seen to be very thick, the somatopleure being in the form of a simple columnar epithelium and the splanchnopleure being in the form of a cuboidal or squamous epithelium (Figs. 2.53 to 2.55). This occurs in the lateral plate mesoderm, i.e., in the region associated with (or surrounding) the embryonic axis (Fig. 2.49).

(Text continues on page 89)



Figure 2.53

Scanning electron micrograph of the area pellucida of a stage 7-8 blastoderm. The mesodermal cells are seen to have become aligned to form two adjacent layers of cells. The dorsal layer of these cells (S) is the fore-runner of the somatopleure. The ventral layer of these cells (SP) is the fore-runner of the splanchnopleure. Clefts (C) develop between these two layers and give rise to the coelom. The rounded 'golf ball' body (G) ventral to the developing splanchnopleure is a primordial germ cell (see Section 2.3.5).

EC, ectoderm; EN, endoderm; G, primordial germ cell;
S, developing somatopleure; SP, developing splanchnopleure.

x 3410



Figure 2.54

Scanning electron micrograph of the area pellucida of a stage 9 blastoderm. The somatopleure (S) and the splanchnopleure (SP) have become separated from each other, resulting in the formation of the extraembryonic coelom (C).

C, coelom; EC, ectoderm; EN, endoderm; S, somatopleure; SP, splanchnopleure.

x 2327

Laterally in the AP the coelom linings are seen to thin out and form simple squamous epithelia (Fig. 2.49). The simple squamous coelomic linings of the AP extend to the pellucida-opaca junction where the coelomic linings of the AP become continuous with the coelomic linings of the AOV.

In the AP, as in the AOV, the somatopleuric lining of the coelom is a single layer of cells which underlies the ectoderm (Figs. 2.53 to 2.55). Likewise, the splanchnopleuric lining of the coelom is a single layer of cells which covers the underlying endoderm and the developing blood vessels (Figs. 2.53 to 2.55). Indeed, the splanchnopleure is seen to surround the blood vessels of the AP so closely that it takes on a scalloped

(Text continues on page 91)







Figure 2.55

Light micrographs of the area pellucida of a stage 11 (top and middle micrographs) and a stage 12 (bottom micrograph) blastoderm. The somatopleuric (S) and splanchnopleuric (SP) linings of the coelom (C) develop from two layers of cells which become separated from each other by the formation of clefts (C). As the splanchnopleure (SP) surrounds the developing blood vessels (L) it takes on a scalloped appearance. Numerous stages in the formation of the blood vessels (L) from the angioblasts (A) are evident. The developing blood vessels (L) are empty of intravascular blood cells.

A, angioblasts; C, coelom; EC, ectoderm; EN, endoderm; L, vessel lumen; S, somatopleure; SP, splanchnopleure.

Top x 417; Middle x 417; Bottom x 667

appearance (Fig. 2.55).

2.3.4 The development of the first embryonic erythrocytes of the chick blastoderm

2.3.4.1 Background

Section 2.3.2 above outlined the cellular events involved in the morphogenesis of the blood-islands in the AOV of the early chick blastoderm, up to stage 12. As described in that section, the yolk sac blood-islands differentiate into the blood vessels and the first developing blood cells of the chick blastoderm (Murray, 1932; Edmonds, 1966; Gonzalez-Crussi, 1971; Small and Davies, 1972).

During normal chick embryogenesis two morphologically distinguishable erythroid cell types (Lucas and Jamroz, 1961; Edmonds, 1966; Small and Davies, 1972) with electrophoretically distinct haemoglobins (Bruns and Ingram, 1973a,b; Brown and Ingram, 1974; Mahoney *et al*, 1977) appear in sequence in the embryonic circulation. The first series is the primitive erythrocytes and the second series is the definitive erythrocytes (see reviews by Wilt, 1967; Bruns and Ingram, 1973a).

The first series, the primitive cells, develop as a cohort and are the only type of erythrocyte present in the circulation during the first five days of incubation (see Lucas and Jamroz, 1961; Bruns and Ingram, 1973a). With respect to morphology, these cells tend to be at the same maturation stage at the same time (Lucas and Jamroz, 1961; Edmonds, 1966; Small and Davies, 1972). Thus, on day two, when the blood-island cells are first becoming liberated into the yolk sac blood vessels (Fig. 2.56), the population consists predominantly of proerythroblasts and basophilic erythroblasts (Lucas and Jamroz, 1961; Edmonds, 1966; Small and Davies, 1972). On the third day the population consists mainly of early- and mid polychromatophilic erythroblasts, on the fourth of late polychromatophilic erythroblasts, and on the fifth day, of the immature primitive erythrocytes (Lucas and Jamroz, 1961; Edmonds, 1966; Small and Davies, 1972). During this period of maturation the cells continue to divide, undergoing an average of six successive

(Text continues on page 93)

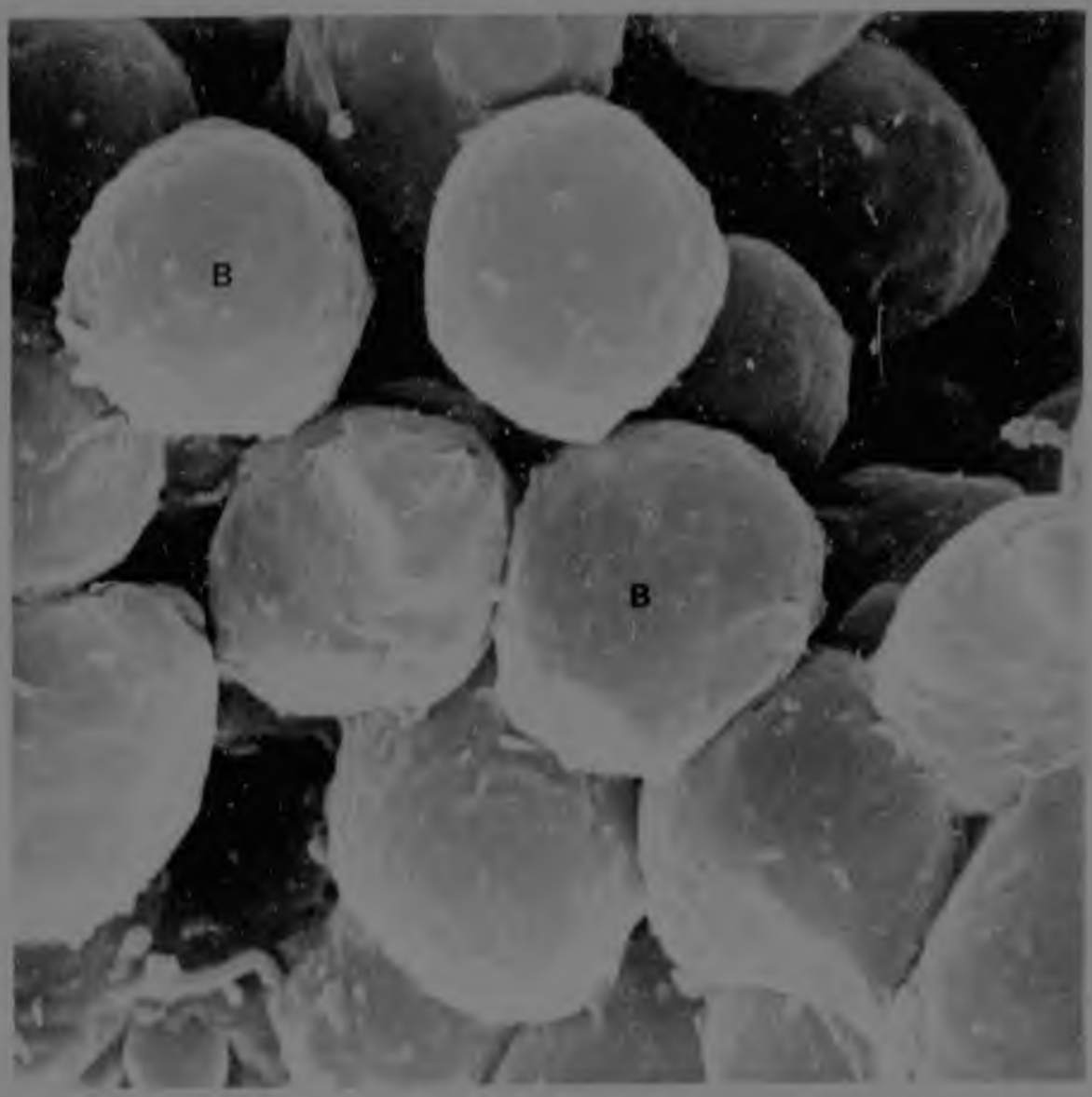


Figure 2.56

Scanning electron micrograph of the cells (B) of an intra-vascular blood-island in the area opaca vasculosa of a stage 10-11 blastoderm. At this stage the individual cells of the blood-island (B) are more or less spherical with numerous short processes extending from their surfaces. These cells will soon become liberated into the vessel lumen as the first developing blood cells.

x 16,120

divisions (Weintraub *et al*, 1971; Campbell *et al*, 1971). These cells reach a maximum in cell number at eight days of incubation, and then decrease in number until they are no longer detectable in the circulation by sixteen days (see Lucas and Jamroz, 1961; Bruns and Ingram, 1973a).

By the end of the fifth day, however, the second or definitive series of erythrocytes begins to make its first appearance in the circulation (see Bruns and Ingram, 1973a). In contrast to the primitive erythrocytes which mature as a cohort, the definitive series is a sequestered population of cells, there being a number of different maturational stages of these cells present simultaneously (see Lucas and Jamroz, 1961; Bruns and Ingram, 1973a). Over the next ten to eleven days the definitive cells increase in proportion from 1-4% of the circulating erythroid elements at the end of day five, to 50% of the circulating cells at day seven, to 100% of the erythroid line by day 16 (see Bruns and Ingram, 1973a).

Thus, three distinct stages of erythropoiesis can be recognised during the development of the chick embryo: the first between 1,5 and 5 days when only primitive erythroid cells are found in the embryonic circulation; the second, between 6 and 16 days when both primitive and definitive erythroid cells exist; and the third, after 16 days when only definitive erythroid cells are found in the embryonic circulation (see Bruns and Ingram, 1973a). The number and types of electrophoretically separable haemoglobins produced during development are found to correlate with these three stages of erythropoiesis, haemoglobins E, P and ? being associated with the primitive cells and haemoglobins A and D being associated with the definitive cells (Bruns and Ingram, 1973a,b; Brown and Ingram, 1974; Mahoney *et al*, 1977).

Although it was previously held that the yolk sac blood-islands were probably the sole sources of all these embryonic blood cells (see Metcalf and Moore, 1971), it has recently been shown that the yolk sac blood-islands provide most of only the primitive erythrocytes, the majority of the definitive erythrocytes being seeded from within the body of the embryo itself (Beaupain *et al*, 1979 - see Discussion, Section 2.4.5).

2.3.4.2 Scanning electron microscopic observations of 3-day and 5-day blood cells

In the present study, SEM observations were made of blood cells isolated from 3-day (Figs. 2.57, 2.58) and 5-day (Figs. 2.59 to 2.61) chick blastoderms. In line with the finding of Bruns and Ingram (1973a,b) the cells observed in the present study would therefore have been successive stages in the morphogenesis of the primitive erythroid line.

In blood cell preparations from 3-day chick blastoderms the following cell types are evident (Figs. 2.57, 2.58):

i. Spherical rough-walled cells (cell type a) and mitotic cells in telophase;
ii. Circular, flat disc-shaped cells with rough surfaces (cell type b). Some of these cells have processes projecting from their surfaces;
iii. Circular to oval flat cells with smooth surfaces (cell type c). Some of these cells exhibit small holes in their surfaces which probably correspond to the pinocytotic vesicles observed under the TEM by Edmonds (1966) and Small and Davies (1972). As suggested by these previous authors, pinocytosis may represent at least one mechanism by which these cells take up extracellular materials, possibly even ferritin.
iv. Crescent-shaped cells with smooth surfaces (cell type d).

No attempt was made to identify rigidly the different stages. However, according to the studies of Lucas and Jamroz (1961), Edmonds (1966) and Small and Davies (1972), these cells probably represent basophilic erythroblasts and early- and mid polychromatophilic erythroblasts. The observations of mitotic cells in telophase are in line with the fact that these developing erythroid cells continue to divide as they mature.

Embryonic thrombocytes, which have the same function as mammalian blood platelets, should also be observable in the circulation of the early chick blastoderm (Sugiyama, 1926; Lucas and Jamroz, 1961; Edmonds, 1966). These cells are reported to make their first appearance in the circulation soon after the

appearance of the primitive erythroid series (see Lucas and Jamroz, 1961). These cells, being free of haemoglobin, are reported to be clearly distinguishable from the developing erythrocytes by benzidine staining (Lucas and Jamroz, 1961). However, no attempt was made to distinguish them from the developing erythroid cells in the present SEM study.

It should be noted that none of the blood corpuscles observed in the present micrographs would correspond to white blood corpuscles. The differentiation of the white blood corpuscles only begins to occur at a much later stage of development (from about day 9 at the earliest) when the intraembryonic haemopoietic organs start to become functional (see Metcalf and Moore, 1971). Furthermore, Lucas and Jamroz (1961) state that "The erythrocytes and the thrombocytes are the only two cell types normally present in the circulating blood of the chick embryo. All others that develop within hematopoietic organs are held there until after hatching." (Lucas and Jamroz, 1961 page 141).

In blood cell preparations from 5-day chick blastoderms the following cell types are evident (Figs. 2.59 to 2.61):

i. Most of the erythroid cells are now more uniform in shape, tending to be round, flat and disc-shaped (cell type e). In line with the studies of Lucas and Jamroz (1961), Edmonds (1966) and Small and Davies (1972), these cells probably represent the immature primitive red blood corpuscles.
ii. A small number of crescent-shaped cells (cell type d) which may represent the late polychromatophilic erythroblasts;
iii. Spherical cells and mitotic cells in telophase (cell type a) which are probably the primitive erythroid cells undergoing mitosis, although some of them may also be the thrombocytes.

(Text continues on page 101)



Figure 2.57

Scanning electron micrographs of blood cells from 3-day chick blastoderms. Most of the cells have undergone changes in cell shape. These cell morphologies probably represent a number of successive erythroblastic stages during the differentiation of the primitive series of blood corpuscles. Some of the cells exhibit small holes (arrows) which probably represent evidence of pinocytotic activity.

a,b,c,d. stages in the differentiation of the blood cells (see text); arrows, pinocytotic vesicles.

x 3036

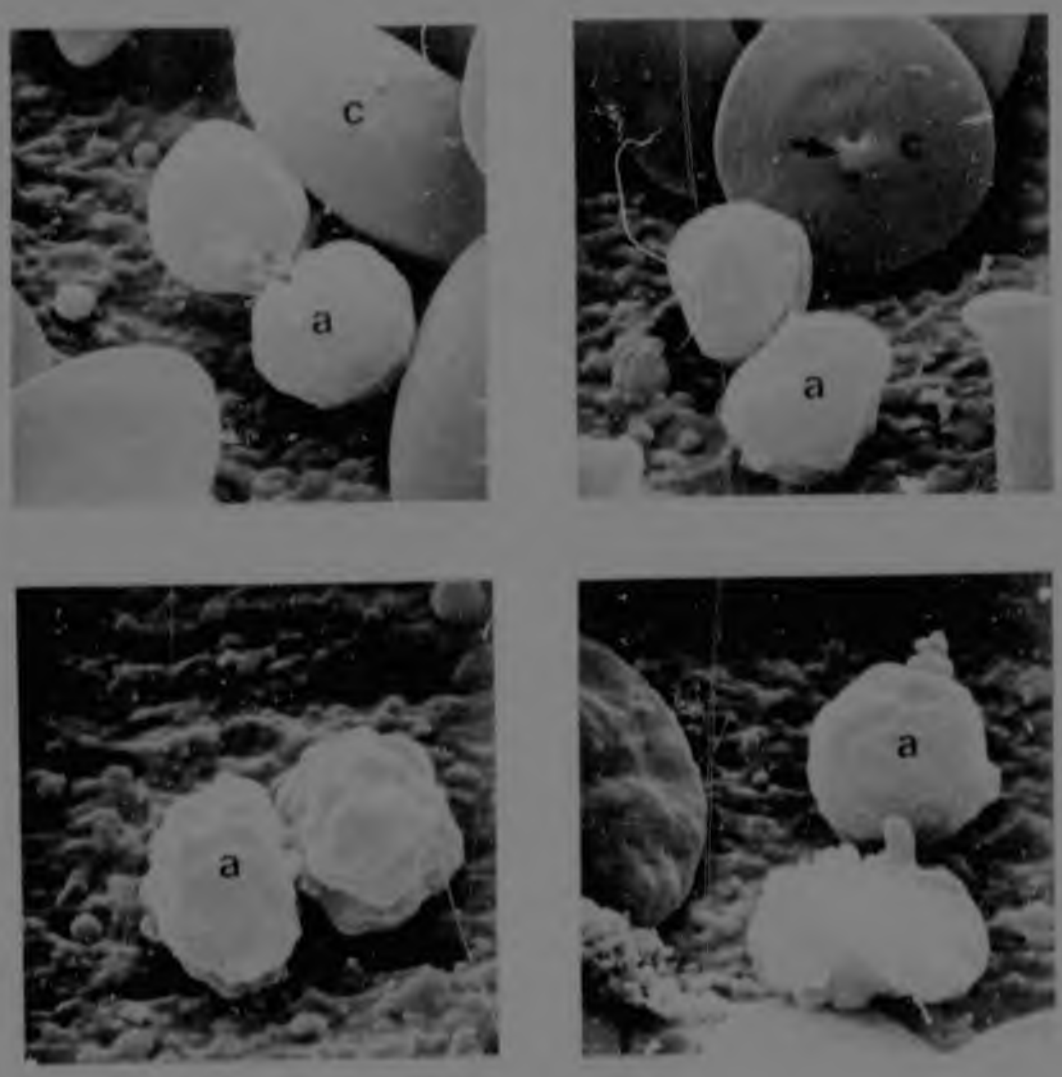


Figure 2.58

Scanning electron micrograph of blood cells from 3-day chick blastoderms to show the mitotic cells in telophase (a). As the primitive blood cells circulate and mature, they undergo an average of six successive mitotic divisions (Weintraub *et al*, 1971). Also present in the micrographs are flat circular to oval cells (cell type c) which represent a stage in the maturation of the primitive erythroid line.

a, primitive erythroid cells in mitosis; c, developing primitive erythroblast; arrow, pinocytotic vesicle.

x 7590



Figure 2.59

Scanning electron micrographs of blood cells from 5-day chick blastoderms. Most of the cells are now more uniform in shape (e), tending to flat and disc-shaped. These cells probably represent the immature primitive erythrocytes. A small number of mitotic cells (a) are also seen.

a, mitotic cells; e, immature primitive erythrocytes.

x 4268

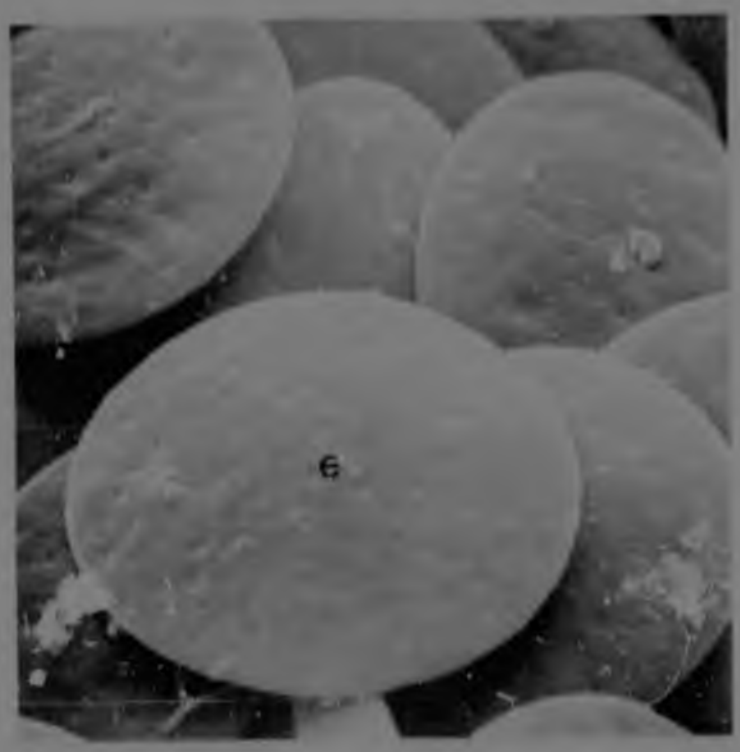


Figure 2.60

Scanning electron micrograph of blood cells from 5-day chick blastoderms. Most of the cells are now flat and disc-shaped (e). These cells probably represent the immature primitive erythrocytes. In the top micrograph they are flatter than those in the bottom micrograph. A crescent-shaped cell (d) is also present in the bottom micrograph.

d, crescent-shaped primitive erythroblast;
e, immature primitive erythrocytes.

x 10,670

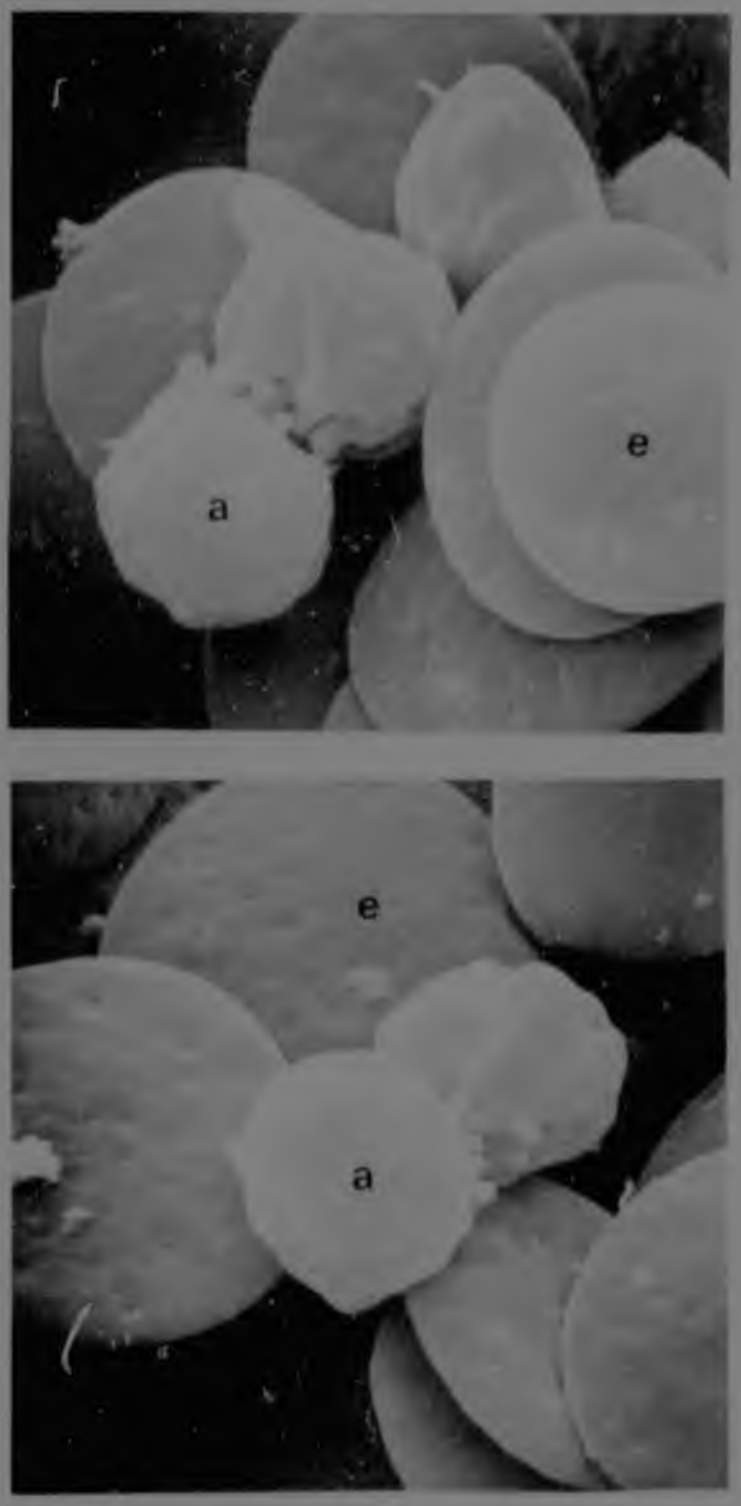


Figure 2.61

Scanning electron micrographs of blood cells from 5-day chick blastoderms. Most of the cells are flat disc-shaped cells (e), representing the immature primitive erythrocytes. Also present in the micrographs are the primitive erythrocytes in mitosis (a).

a, mitotic cells; e, immature primitive erythrocytes.

x 10,670

2.3.5 Observations of the primordial germ cells

The primordial germ cells (PGCs) of the chick blastoderm originate in the opaca endoderm of the so-called 'germinal crescent', a crescent-shaped region which borders the anterior and anterio-lateral part of the AP (Swift, 1914; Meyer, 1964; Clawson and Domm, 1967; Fujimoto *et al*, 1976a,b; Lee *et al*, 1978). From the primitive streak stage these cells begin to separate from the endoderm and most of them later enter the developing blood vessels of the yolk sac vascular system. Most of the PGCs are then transported via the blood vessels into the body of the embryo, from where they finally move into the gonadal anlage itself (Swift, 1914; Meyer, 1964; Clawson and Domm, 1967; Fujimoto *et al*, 1976a,b).

During the course of the present investigation PGCs were often observed among the differentiating mesodermal cells of the yolk sac vascular system, and they were often found to become incorporated into the lumens of the developing yolk sac blood vessels (Figs. 2.9, 2.34, 2.53, 2.62 to 2.65). In agreement with the descriptions of previous authors, the PGCs were readily distinguishable from the surrounding mesodermal cells of the developing yolk sac vascular system by their large size (averaging 11-20μm in diameter at the earlier stages (Figs. 2.34, 2.53, 2.62, 2.65) and 9-16μm in diameter at the later stages (Figs. 2.66, 2.67)), by their large round nucleus (8-10μm in diameter) occupying an eccentric location in the cell (Figs. 2.66, 2.67), and by the presence of considerable amounts of yolk ('nonglycogen PAS-positive granules', 1,5 to 5μm in diameter (Clawson and Domm, 1967)) in their cytoplasm during the stages of their separation from the endoderm and their early migration in the blood vessels (Meyer, 1964; Clawson and Domm, 1967; Fujimoto *et al*, 1976a,b) (Figs. 2.9, 2.34, 2.53, 2.62 to 2.65). In the SEM sections of the present study these granules were seen to bulge outwards under the cell membrane (Figs. 2.9, 2.34, 2.53, 2.62 to 2.64) and in the present light microscopic sections the PGCs were seen to be packed with eosinophilic granules (Fig. 2.65) of the same size range. As reported by the previous authors, these yolk granules were found to have become dissipated during the later stages (Clawson and Domm, 1967; Fujimomo *et al*, 1976a,b) and this was accompanied by

(Text continues on page 103)

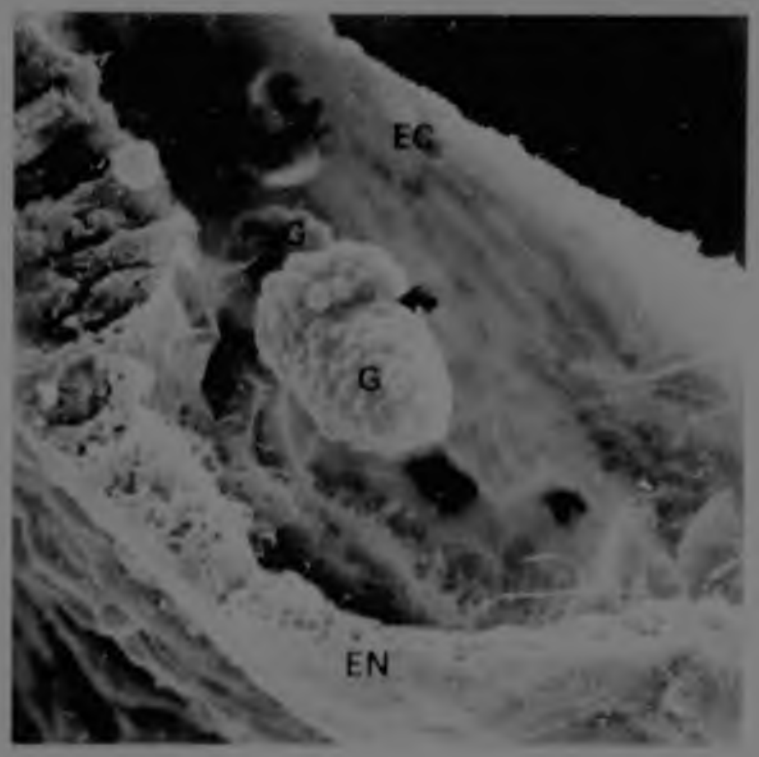


Figure 2.62

Scanning electron micrograph of the pellucida-opaca border of a stage 5 chick blastoderm prior to the migration of the mesodermal sheet into the AOV. Two large yolk-laden primordial germ cells (G) are present in the space between the ectoderm (EC) and endoderm (EN). It is possible that the nearer of the two cells may be in the process of mitosis since there appears to be a constriction (arrow) in the middle of the cell.

EC, ectoderm; EN, endoderm; G, primordial germ cell; arrow, constriction in cell.

x 1552



Figure 2.63

Scanning electron micrograph of the area opaca vasculosa of a stage 7 blastoderm. The mesodermal cells (M) in the space between the ectoderm (EC) and endoderm (EN) are in the process of differentiating into the yolk sac vascular system. Present among the differentiating mesodermal cells are two large yolk-laden 'cells' (G). The cell on the left corresponds in size and appearance to a primordial germ cell. However, the 'cell' on the right is extremely large and irregularly shaped, appearing as if it has become folded upon itself (see also Fig. 2.64). Whether or not this cell is a single primordial germ cell is difficult to say with certainty from the present micrograph.

EC, ectoderm; EN, endoderm; G, yolk-laden 'cells' (PGCs); M, mesodermal cells.

x 1164

a decrease in the size of the PGCs from the size range of 11-20μm to the size range of 9-16μm, as reported by Clawson and Domm (1967) (Figs. 2.66, 2.67).

In one case an extremely large irregularly shaped yolk-laden 'cell' was observed among the differentiating mesodermal cells in the AOV of a stage 7 blastoderm (Figs. 2.63, 2.64, lower micrograph).

(Text continues on page 106)





Figure 2.64 (See legend overleaf)

EC, ectoderm; EN, endoderm; G, yolk-laden 'cells' (PGCs); M, mesodermal cells.

x 2328

Figure 2.64 (See photographs on previous page)

Higher powered scanning electron micrographs of Figure 2.63 to show the yolk-laden 'cells' (G) in more detail. The cell in the top micrograph corresponds in size and appearance to a primordial germ cell. However the 'cell' in the bottom micrograph is extremely large and irregularly shaped. Whether or not this 'cell' is a single primordial germ cell cannot be said with certainty in the present study. One suggestion is that this 'cell' may be composed of two or more primordial germ cells which are very close together, and which may even be the products of mitosis. However this is pure conjecture.

EC, ectoderm; EN, endoderm; G, yolk-laden 'cells' (PGCs); M, mesodermal cells.

x 2328

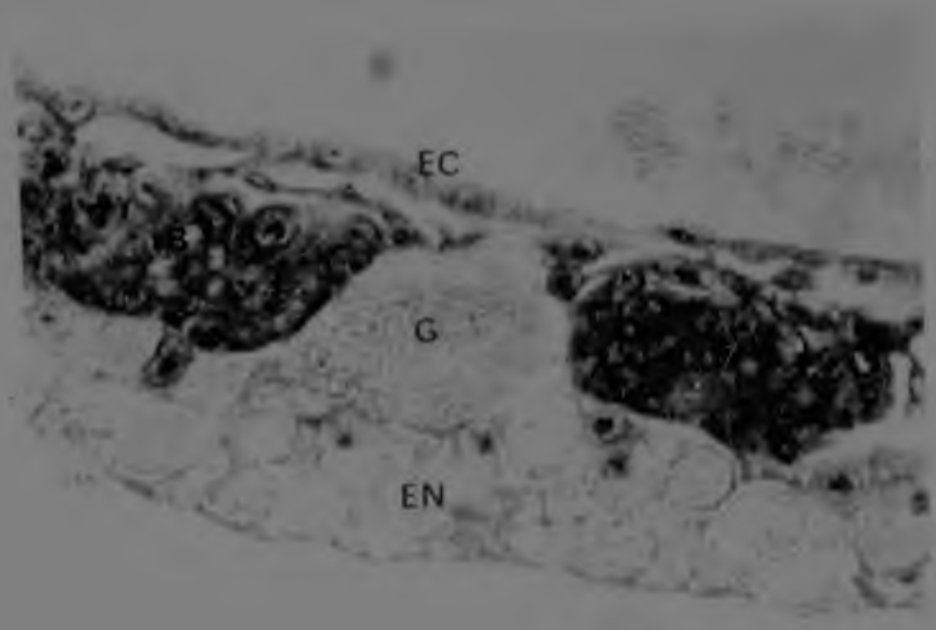


Figure 2.65

Wax embedded light microscopic section of the area opaca vasculosa of a stage 6 blastoderm. A primordial germ cell (G) with its cytoplasm characteristically packed with eosinophilic yolk granules is seen on the surface of the yolk sac endoderm (EN) between two blood-islands (B) (see also Figure 2.9, page 33). The nucleus, being eccentric, is not shown in the present section.

B, blood-islands; EC, ectoderm; EN, endoderm; G, primordial germ cell.

x 1419

This 'cell' appeared to be folded up on itself and multi-lobed. Whether or not this 'cell' is a single primordial germ cell was difficult to say with certainty in the present study. One suggestion is that this 'cell' may be composed of more than one PGC which are very close together, or are daughter cells which have not completely separated after mitosis. PGCs have previously been observed to undergo mitosis (Clawson and Domm. 1967; Fujimoto *et al*, 1976a). However, it is very difficult to interpret the situation clearly from the present SEM micrograph (Figs. 2.63, 2.64, lower micrograph).

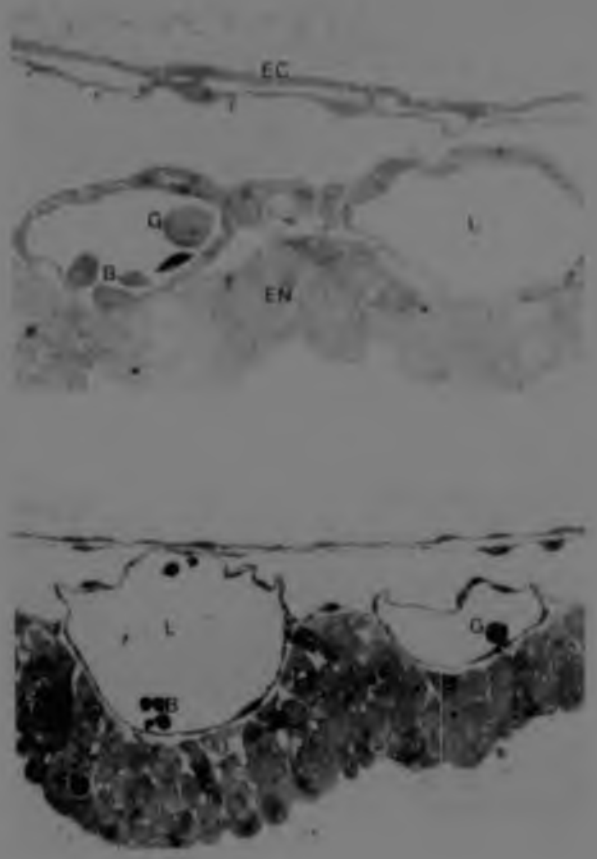


Figure 2.66

Plastic embedded light microscopic sections of blood vessels (L) in the area opaca vasculosa of stage 12-13 blastoderms. Present in the lumens of the vessels (L) are intravascular blood cells (B) and primordial germ cells ( ).

B, blood cells; EC, ectoderm; EN, endoderm;
G, primordial germ cell; L, vessel lumen.

Top x 711; Bottom x 356

The micrographs in the present study show PGCs which were observed in the following situations: Figure 2.62 shows yolk-laden PGCs at the pellucida-opaca boundary of a stage 5 blastoderm prior to the migration of the mesodermal sheet into the AOV. Figures 2.63 and 2.64 show the AOV of a stage 7 blastoderm which has been invaded by the migrating mesodermal sheet. In these micrographs a PGC and a large irregularly shaped yolk-laden 'cell' (PGCs?) are present amongst the differentiating mesodermal cells.

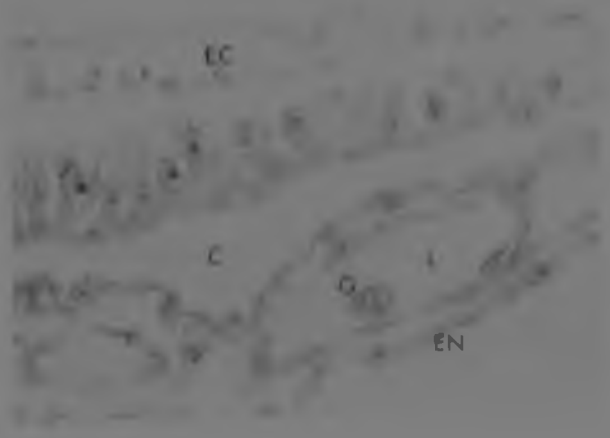


Figure 2.67

Wax embedded light microscopic section of the area pellucida of a stage 11-12 blastoderm. A primordial germ cell (G) is seen in the lumen (L) of a blood vessel. Its large, round eccentrically placed nucleus is clearly evident. The yolk granules which previously filled the cytoplasm (see Fig. 2.65) have now become dissipated.

C, coelom; EC, ectoderm; EN, endoderm;
G, primordial germ cell; L, vessel lumen.

x 711

In Figures 2.9 (page 33) and 2.65 PGCs are seen in the AOV in association with the yolk sac blood-islands. In Figure 2.34 (page 64) a PGC is seen to have become incorporated within the lumen of a developing yolk sac blood vessel. In that micrograph the PGC is observed within the newly formed vessel lumen at a stage prior to the dissociation of the prospective blood cells. PGCs were also observed within the lumens of blood vessels at later stages of development when the circulation of the blood had already commenced (Figs. 2.66, 2.67). These cells were found in the vessels of both the AOV (Fig. 2.66) and the AP (Fig. 2.67), indicating the translocation of these cells from the AOV towards the body of the embryo. In agreement with Clawson and Domm (1967) and Fujimoto *et al* (1976a,b) the PGCs at these later stages appear to have lost their yolk granules (compare Figs. 2.66 and 2.67 with Fig. 2.65).

The subsequent migration of the PGCs into the body of the embryo and their entry into the gonadal anlage was not followed in the present study.

2.4 Discussion

The SEM and light microscopic study carried out in the present chapter has afforded an excellent means of following the cellular organization and patterning involved in the transformation of a migrating population of mesodermal cells into an organized system of cellular tubes, free cells, and large cell-lined spaces. The events involved in this cellular patterning include:

1. The invagination of the primary mesenchymal cells from the epiblast into the space between the epiblast and hypoblast;
2. The centrifugal migration of these cells through the AP into the AOV to form the mesodermal layer (this excludes the presumptive endodermal cells which enter into the underlying hypoblastic layer of the AP to form the embryonic endoderm);
3. The clumping together of some of the mesodermal cells in the AOV and AP to form the blood-islands in the AOV and the angioblasts in the AP;
4. (a) The morphogenesis of the surface cells of the opaca blood-islands into a flattened endothelial lining, and the differentiation of the central cells of the opaca blood-islands into the intravascular blood cells;
   (b) The transformation of the angioblasts in the AP into hollow blood vessels;
5. The joining up of the blood vessels into a plexus;
6. The organization of the rest of the mesodermal cells to form the linings of the extraembryonic coelom.

Whereas many aspects of the present investigation overlap with aspects of previous descriptive studies in the literature, the present analysis also fills in some of the gaps in these descriptions, and furthermore, attempts to resolve the discrepancies in the descriptions of blood-island differentiation which have stemmed from some of the older literature (Sabin, 1917, 1920; Sugiyama, 1926; Houser *et al*, 1961).

2.4.1 The origin of the primary mesenchymal cells

As described in Section 2.3.1, the mesodermal cells which give rise to the yolk sac vascular system originate in the epiblast of the early chick blastoderm. The first step in the creation of

the cellular patterning involves the invagination of these cells through the primitive streak into the space between the epiblast and hypoblast. This translocation of cells from an 'outside' position (at the surface of the blastoderm) to an 'inside' position (in the interior of the blastoderm) is accompanied by changes in cell shape. As described in the present SEM study, and in agreement with the TEM observations of Balinsky and Walther (1961), it is found that whereas the epiblast lateral to the primitive streak is in the form of a simple columnar epithelium, in the region of the primitive streak the epiblastic cells are seen to have become progressively more elongated and flask-shaped. The apical ends of the flask cells are seen to be constricted and they form long narrow bottle-necks which maintain their attachment at the surface of the epiblast. Meanwhile the basal ends of the flask cells have become expanded and they are seen to extend down into the interior of the blastoderm. Finally the flask cells lose contact with the surface of the epiblast and they become totally displaced into the space between the epiblast and hypoblast. Throughout this period the flask cells and the newly invaginated primary mesenchymal cells continue to make contact with each other by means of cellular processes.

This change in cell-shape which accompanies the invagination of these cells through the primitive streak of the chick blastoderm was first described by Balinsky and Walther (1961), and it parallels the observation of Holtfreter (1943) of the formation of bottle cells during gastrulation in the amphibian embryo. The formation of flask cells in the chick primitive streak has also subsequently been described by Granholm and Baker (1970) and Solursh and Revel (1978). This change in cell shape is the visual manifestation of a change in cell behaviour which occurs when a regular epithelium, made up of closely packed columnar cells, becomes transformed into a loose irregular meshwork of migratory mesenchymal cells.

A further factor in the transformation of this regular epithelium into a loose migratory mass of cells is the formation of large extracellular spaces between the epiblast and hypoblast through which the cells can migrate. Solursh (1976) has shown that large amounts of glycosaminoglycans (GAGs), especially hyaluronate, are being synthesized by the cells of the blastoderm during these stages of invagination and migration. Since dilute solutions of hyaluronate have a hydrated

volume 10^3 larger than that of the unhydrated state (see Laurent, 1970), Solursh (1976) suggests that the timely secretion of hyaluronate into the extracellular space, followed by its extensive hydration, could result in the expansion of the extracellular spaces necessary for the invagination and migration of the primary mesenchymal cells. Evidence in favour of this proposal comes from the finding of Fisher and Solursh (1977) that the removal of hyaluronate during these stages (with hyaluronidase treatment) causes a decrease in the volume of the extracellular spaces and the clumping together of the mesenchymal cells.

As described above (Section 2.3.1), most of the primary mesenchymal cells move centrifugally through the AP and AOV to form the extraembryonic mesodermal layer of the blastoderm. These cells will subsequently become organized to form the blood vessels, blood cells, and coelomic linings of the yolk sac vascular system. However, not all the invaginating mesenchymal cells are destined to give rise to the mesodermal yolk sac vascular system. Some of the invaginating cells enter into the underlying hypoblastic layer of the AP and give rise to the embryonic endoderm (Hunt, 1937; Rosenquist, 1966; Nicolet, 1967, 1970). These latter cells, although not clearly distinguishable from the rest of the mesodermal cells histologically, have been shown by autoradiographical methods to give rise to the endoderm of the AP (Rosenquist, 1966; Nicolet, 1967, 1970).

2.4.2 The centrifugal migration of the primary mesenchymal cells

As found in the present study, and in agreement with the observations of Balinsky and Walther (1961), Trelstad *et al* (1967), Granholm and Baker (1970), Ebendal (1976) and Revel and Solursh (1978), the primary mesenchymal cells are typical migratory cells, being irregularly shaped with numerous finger-like filopodia and broader lamellipodia extending from their surfaces. These cells migrate posteriorly, laterally, and antero-laterally from the primitive streak, forming an irregular sheet of cells which spreads centrifugally through the AP into the AOV (see Rosenquist, 1966; Nicolet, 1967, 1970). Once in the AOV, the antero-lateral wings of the mesodermal sheet undergo a major change in the direction of cell movement, spreading anteriorly and anteromedially around the anterior border of the AP to meet each other medially on the anterior border of the AP (see Fig. 1.3, page 5). In agreement with previous authors (Trelstad *et al*, 1967; Revel, 1974; Ebendal, 1976;

Revel and Solursh, 1978) the migratory mesenchymal cells continue to make contact with each other as well as with the overlying epiblastic layer and the underlying hypoblastic layer by means of their filopodia and lamellipodia. As suggested by Ebendal (1976), these cells probably use the surfaces of neighbouring mesenchymal cells, and the under-surface of the epiblast and the upper-surface of the hypoblast as substrata for their outward migration.

Revel and Solursh (1978), in a SEM investigation of the orientation of the individual mesodermal cells in the sheet, reported that there was a lack of consistent cell orientation of the individual cells with respect to the directions of expansion of the sheet. According to these authors, the mesodermal cells appeared to move towards either the periphery or the midline, as well as towards either the hypoblast or the epiblast. These authors identified the leading, forward-moving ends of the cells as the regions bearing the lamellipodia and filopodia. In terms of this definition of the leading end of the cell, the observations of cell orientation in the present SEM study are in agreement with those of Revel and Solursh (1978) that there is a lack of consistent cell orientation of the mesodermal cells in the sheet.

Revel and Solursh (1978) therefore suggest that the centrifugal movement of the mesodermal sheet does not involve the arrow-like migration of its individual cells towards the periphery, but rather that the expanding sheet of cells moves as a unit. According to these authors, ". . . while the net result of cell movement may be a displacement of cells from the midline towards the periphery of the embryo, this could be achieved not by a precisely orchestrated, soldier-on-parade-like movement of individuals, but rather by a series of displacements and cell divisions, the net effect of which would be the translocation of cells laterally simply because the margin of mesenchyme keeps on expanding." (Revel and Solursh, 1978 page 1042).

The question then arises as to what causes, or guides, the pathways of migration of the mesodermal sheet.

A number of models have been proposed to explain directional cell movement. These models, formulated mainly from observations of cells in culture, include (i) contact inhibition, (ii) chemotaxis, (iii) contact guidance, and (iv) gradients in adhesivity of the substratum (see review

by Trinkaus, 1976). Although these models are discussed separately hereunder, it is probable that a combination of these (and/or other) factors may be responsible for the directionality of cell movement.

i. Contact Innibition

A form of *in vitro* cell behaviour considered by many to provide a good model for the movement of cell streams or sheets *in vivo* is contact inhibition of locomotion. This phenomenon, first observed in the haemocytes of the king crab by Loeb (1921) and subsequently described in cultures of fibroblasts by Abercrombie and Heaysman (1954) involves the following:

When the leading edge of one cell collides with another cell, the movement of the first cell stops in the direction that has brought it into collision, and its movement then tends to be diverted into a new direction by the formation of a new leading edge (see Trinkaus, 1976; Abercrombie, 1980). By this means cells tend to move away from more densely populated regions into less densely populated regions. However, the movement of individual cells is directional only relative to the last cell that they contacted. They always move away from this cell into available free space. Contact inhibition of cell movement can therefore give directionality to the movement of cell populations only when the cell source is a single mass of many cells. If the cells are dissociated and seeded randomly as a suspension, they will also show contact inhibition as they collide, and will also tend to move away from each other, but there will be no overall directionality to their movement.

This model of contact inhibition has been suggested by Trelstad *et al* (1967) (see also Hay, 1968) to explain the centrifugal migration of the primary mesenchymal cells from the densely populated primitive streak. In terms of this model, the invaginating cells from the primitive streak would make contact with the underlying cells (either neighbouring mesenchymal cells or the underlying hypoblast) and would therefore be diverted to migrate in a lateral direction between the epiblast and hypoblast. During this migration the mesenchymal cells would continue to make filopodial and/or lamellipodial contacts with neighbouring mesenchymal cells and with the epiblastic and hypoblastic layers. This would result in further contact inhibitions, and therefore further changes in the directions of movement of the individual cells. Only those cells at or near the periphery of the sheet would have substantial free space into

which to move. This would tend to be in a centrifugal direction, i.e., away from the primitive streak. As these cells moved out, those behind them would have more space into which to move, and they too would move out. However, although the cell movement would, on the average, be directional, i.e., away from the highly populated region towards the peripheral more sparsely populated regions, at any given moment the individual cells may not be moving directionally relative to the direction of expansion of the mesodermal sheet. This is because the direction of movement of the individual cells would tend to be away from the last cell they have contacted, into any available free space. This free space, at any given moment, could be above or below, behind or in front of the cell they have just contacted.

This explanation of directional cell movement in terms of the model of contact inhibition would be in line with the observations of Revel and Solursh (1978) of a lack of consistent cell orientation of the individual mesodermal cells which migrate out from the streak. However, this lack of consistent cell orientation could also be a feature of chemotaxis - see below.

ii. Chemotaxis

The chemotactic movement of cells towards an attractant would seem to be an ideal model for the directional movement of the mesodermal cells towards the AOV. Well-known examples of this type of directionality of movement are the attraction of leucocytes to bacteria (Ramsey, 1972a,b) and the streaming of slime mould cells towards a centre (see Bonner, 1947, 1971; Gerisch, 1968; Alcantara and Monk, 1974; Gross *et al*, 1976; Loomis, 1979).

Ramsey (1972a,b) made time-lapse cinemicrographical recordings of the locomotion of human polymorphonuclear leucocytes and showed that the vast majority of the cells exposed to a gradient of the attractant (a culture of the bacterium *Staphylococcus albus*), moved towards the source of the attractant. However, although the chemotactic movement of these cells was overall directional, there was nevertheless a certain amount of variable behaviour on the part of the individual cells. Tracings of cell paths revealed that the movements of the individual cells towards the attractant were often tortuous and indirect (Ramsey, 1972a). Thus, at any given moment the individual cells would not necessarily be moving

directly towards the bacteria. Ramsey (1972b) found, furthermore, that the lamellipodia of the cells tended to be extended at random from all sides of the cells, but that the cells generally tended to move in the direction of the lamellipodia which were facing towards the attractant.

Aggregation in the cellular slime mould *Dictyos l'um disco deum* is also mediated by chemotaxis, the cells being attracted towards a centre by a relayed signal of cyclic AMP (see reviews by Gerisch, 1968; Bonner, 1971; Loomis, 1979). A competent cell receiving a super-threshold concentration of cyclic AMP moves towards the signalling cell(s), and it in turn secretes a pulse of cyclic AMP. This pulse attracts further cells which move towards the latter signalling cell, and they in turn secrete a pulse of cyclic AMP. The unidirectional propagation of the signal is maintained by the production by the cells of the degradative enzyme of cyclic AMP, phosphodiesterase. Thus directional pulses of cyclic AMP are constantly being produced and removed.

The cells respond to these periodical emissions of signals by propagated waves of inward movements. This is clearly seen during the early stages of aggregation as concentric patterns of propagated dark and light bands which spread out from the signalling centres (see Gerisch, 1968; Alcantara and Monk, 1974). Microscopical examinations of these bands show that at any instant the light bands consist of elongated moving cells while the dark bands contain rounded cells with randomly oriented lamellipodia. The light band areas therefore contain the cells which are actively moving towards the signalling centres, whereas the darker band areas contain the cells which have ceased to move in response to the cyclic AMP signal that has just passed them, and which have not yet been stimulated by the next signal (Alcantara and Monk, 1974).

Recent work by Robertson and Gingle has implicated the role of cyclic AMP in the attraction of the migratory cells in the chick blastoderm in a manner similar to its role in the attraction of the cells of *D. discoideum* (Gingle, 1977; Robertson, 1977; Robertson and Gingle, 1977; Robertson *et al*, 1978; Gingle and Robertson, 1979). Reporter and Rosenquist (1972) had earlier shown that there were regional differences in the cyclic AMP, phosphodiesterase and ATP content of stage 5 blastoderms, the hypoblast below the mesodermal forming areas having relatively higher concentrations of these compounds compared with the other regions of the blastoderm.

That chick embryonic cells may indeed be attracted by cyclic AMP has been demonstrated by Robertson and Gingle (1977) and Gingle and Robertson (1979). These workers found that a microelectrode source of cyclic AMP could attract the cells of stage 3-6 chick blastoderms and that this attraction of the cells could even lead to the bending of the embryonic axis towards the source. These authors also showed that the agglutination *in vitro* of the cells of dissociated stage 4-5 chick blastoderms was enhanced by the addition of cyclic AMP to the medium (Gingle, 1977) and furthermore, that cyclic AMP was produced by these cells in response to stimulation by cyclic AMP (Robertson *et al*, 1978). When Gingle (1977) studied the dynamics of this cyclic AMP-enhanced agglutination in terms of the theory of signal relaying (see Gingle, 1976) he concluded that the aggregation of dissociated chick embryonic cells, like the aggregation of the cells of *D. discoideum*, is controlled by a relayed signal of cyclic AMP (Gingle, 1977).

In this regard, examinations of time-lapse films of the movements of the cells in the early chick blastoderm have revealed periodically propagating waves of refractive index change, sometimes clearly associated with cell movements (cited by Robertson and Gingle, 1977). These waves of cell movement are reported to be similar to those observed during the aggregation of the cells of the cellular slime mould (Robertson and Gingle, 1977). It thus seems possible that a chemotactic attraction of the cells in the early chick blastoderm may indeed be a causative factor in the centrifugal expansion of the mesodermal sheet.

iii. Contact Guidance

Contact guidance is the orientated movemert of cells along discontinuities in the substratum. This phenomenon was first described by Harrison (1914), but was intensively studied and named contact guidance by Weiss (1945, 1961). It has been proposed that contact guidance could also serve as a model for directional cell movements in the early chick blastoderm (see below).

During gastrulation in the chick blastoderm, incomplete basal laminae are already present below the ectodermal- and endodermal layers (Trelstad *et al*, 1967; Hay. 1968; Revel, 1974; Ebendal, 1976). As the mesenchymal cells migrate away from the primitive streak they make contact with these basal laminae by means of their cellular processes.

Because of this relationship, Hay (1968) suggested that the surface texture of the basement membranes may help to direct the mesodermal cell movement by contact guidance. Support of this proposal has recently come from Wakely and England (1979) who, in a SEM and histochemical study of the basement membranes in the gastrulating chick blastoderm, report the existence of a pattern of radial and concentric fibrils in the ectodermal basement membrane (see Fig. 2.68). These authors demonstrated the following four well marked zones in the arrangement of these fibrils in the ectodermal basement membrane of blastoderms of stages 3 to 6 (Fig. 2.68):

1. In the posterior and postero-lateral region of the AP, the region through which the mesodermal cells normally migrate during these stages, the fibrils appear as long continuous strands which radiate outwards from the primitive streak towards the lateral pellucida boundary. These strands are intersected by similar long fibrils running concentrically and centred on the primitive streak, the two sets of fibrils together forming a grid (Fig. 2.68).
2. Approximately half-way between Hensen's node and the anterior boundary of the area pellucida the arrangement of the fibrils changes abruptly, and they appear as short, thick, closely-packed rods lying roughly at right angles to the ectodermal surface with no radial fibrils present (Fig. 2.68).
3. A prominent band of fibrils form an arc along the anterior border of the AP, its lateral extremities extending laterally to the level of Hensen's node. The fibrils within the band are long, mainly unbranched, and gently curved. They are at least twice as thick as the fibrils seen in the posterior and posterio-lateral part of the AP (Fig. 2.68).
4. In the anterior part of the AOV beyond the fibrous band fine radial fibrils are observed which extend outwards towards the outer margins of the area opaca (Fig. 2.68).

Wakely and England (1979) propose that the arrangements of these fibrils in the ectodermal basement membrane could provide a contact guidance system, directing the pattern of mesodermal cell migration along the pattern of orientation of the fibrils. According to these authors, the network of radial and concentric fibrils in the posterior and posterio-lateral region of the AP could play a role in the centrifugal migration of the mesodermal cells towards the AOV. "It is likely that the cells advance forward across the fibrillar net, using their filopodia to direct themselves from one

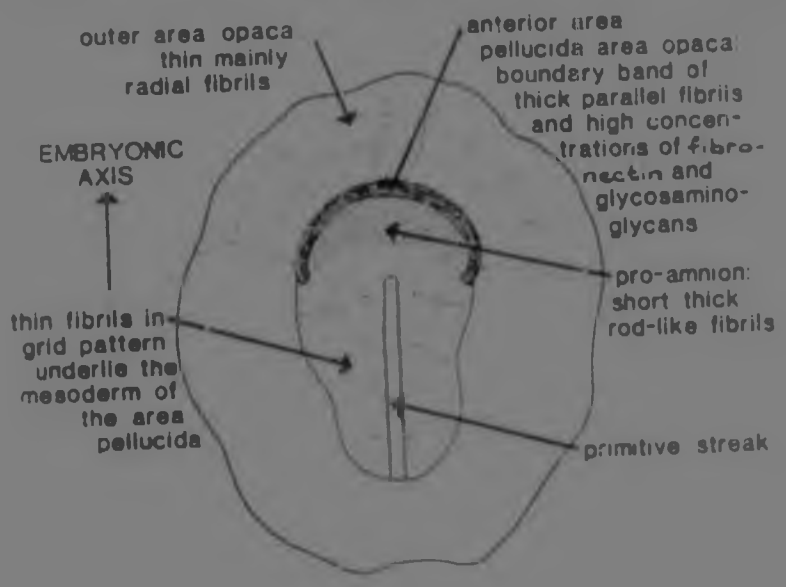


Figure 2.68

Regional variations in the structure of the ectodermal basement membrane of the stage 4 chick blastoderm, as outlined by Wakely and England (1979). The blastoderm is shown as viewed from the ventral surface after the removal of both the endoderm and mesoderm. (After Wakely and England, 1979).

concentric fibril to the next, possibly aligning themselves along the radial fibrils to do so." (Wakely and England, 1979 page 343). From stage 5 the lateral wings of the mesoderm in the AOV begin to spread forward, moving along the anterior boundary of the AP (see Fig. 1.3C page 5). This anterior migration involves a major change in the direction of cell movement, and has been explained by Wakely and England (1979) in terms of the orientation of the thick fibrous band at the anterior border of the AP. According to these authors, "The mesoderm cells could therefore make their initial contact with the band at its posterior end, and then follow the direction of the fibrils of the band around the anterior border of the AP. . . . The physical structure of the band, with its obvious track-like appearance makes it well suited to act as a contact guidance system." (Wakely and England, 1979 page 344). On the outer (area opaca) side of the fibrous band the straight, thin, radiating fibres could be involved in the outward expansion of the mesoderm into the AOV (Wakely and England, 1979).

Wakely and England (1979), using alcian blue staining and immuno-

fluorescence techniques have, furthermore, demonstrated the presence of high concentrations of GAGs and of fibronectin (a cell surface glycoprotein) along the line of the fibrous band at the anterior pellucida border. As suggested previously (Section 2.4.1), GAGs are believed to facilitate cell movement (see Solursh, 1976; Fisher and Solursh, 1977; and the review by Adamson, 1981). Likewise, fibronectin is also believed to play an important role in cell migration (see review by Adamson, 1981). It has been found, for example, that cells deficient in fibronectin are less motile than normal cells and that their movements are less directional. Normal motility can be restored by adding the protein to the culture medium (Pouyssegur *et al*, 1977; Yamada *et al*, 1976, 1978; Ali and Hynes, 1978). As well as facilitating cell locomotion, fibronectin also enhances the adhesion of cells to the substrates on which they move, especially collagen (Pearlstein, 1976; Yamada *et al*, 1976), and increases the tendency of cells to form sheets (Chen *et al*, 1978).

In terms of these findings, it is therefore probable that both the arrangements and the chemical composition of the fibrils in the ectodermal basement membrane play an important role in the migratory pattern exhibited by the mesodermal cells.

iv. Gradient in Adhesiveness of the Substratum

A gradient in differential adhesiveness of the substratum has had much appeal as a model for the directionality of cell movement (Carter, 1965, 1967; Harris, 1973). Carter (1965, 1967) and Harris (1973) observed the movement of cells on gradients of gradually increasing metal density and demonstrated that cells will move up such gradients in a highly directional manner. Carter (1965) termed this phenomenon 'haptotaxis' "(Greek: haptein, to fasten; taxis, arrangement), conveying the idea that the movement of a cell is controlled by the relative strengths of its peripheral adhesions, and that movements directed in this way, together with the influence of patterns of adhesion and cell shape, are responsible for the arrangement of cells into complex and ordered tissues." (Carter, 1965 page 1184). Carter (1965) suggests that cell movement from a less adherent to a more adherent surface is the dominant principle in cell motility, and has attempted to apply the concept of haptotaxis to explain all the other models of directional cell motility, viz. contact inhibition, chemotaxis and contact guidance.

As indicated previously, the above four models explaining the directionality of cell motility (contact inhibition, chemotaxis, contact guidance, and haptotaxis) have been formulated mainly from the observations of tissue culture cells *in vitro* or from fixed histological material. As suggested by Trinkaus (1976) more investigations of the living *in vivo* situation are necessary before a clearer understanding of the guiding principles operative in the living embryo may be obtained.

2.4.3 Pattern formation during the morphogenesis of the yolk sac vascular system

2.4.3.1 Models explaining the patterns of morphogenesis

The models of cell motility described above could well be applied to explain the cellular organization and patterning which occurs during the morphogenesis of the yolk sac vascular system from the cells in the migrating mesenchymal sheet. As described in Section 2.3.2, during the morphogenesis of this system some of the mesodermal cells in the sheet clump together against the yolk sac endoderm to form aggregations of cells, the blood-islands. Meanwhile, the rest of the mesodermal cells in the sheet become organized to form the flattened single-cell-thick coelomic linings which underlie the ectoderm and overlie the endoderm and the blood-islands.

In explaining the behaviour and sorting out of these cells into this cellular patterning, the models of contact inhibition, chemotaxis, contact guidance, and differential adhesivity to the substratum all seem to be of some value. For example, the model of contact inhibition could explain the differences in behaviour between the prospective blood-island cells and the prospective coelomic lining cells. In terms of this theory, it could be implied that the coelomic lining cells exhibit contact inhibition and therefore form monolayers, whereas the blood-island cells show a loss of contact inhibition and therefore pile up into multiple layers of cells. In terms of the model of chemotaxis, it could be suggested that the prospective blood-island cells are chemotactically attracted to the endoderm against which they clump. The principle of contact guidance could be invoked to explain the arrangements and spacing of the blood-islands on the endoderm, and the spreading of the coelomic lining cells over the ectoderm, endoderm and blood-islands. Finally, the concept of haptotaxis could provide an attractive theory to explain why the blood-

island cells clump together against the endoderm and why the coelomic lining cells spread out over the surfaces of the other cell types. In terms of this theory it could be suggested that the blood-island cells clump together against the endoderm because they are more adhesive to the endoderm than to the ectoderm, and are more adhesive to each other than to the rest of the mesodermal cells in the sheet. On the other hand, the coelomic lining cells form cellular linings over the ectoderm, endoderm and blood-islands because they are preferentially adhesive to the surfaces of these cell types than they are to each other.

It must be emphasized that the above explanations are purely hypothetical. It is possible that a combination of these (and/or other) models may also play a role in moulding the patterns of morphogenesis observed. However, experimental evidence is at present still lacking, and an in-depth investigation of the system is therefore required to elucidate the guiding principles of the *in vivo* situation.

2.4.3.2 Gradients and/or polarities in the pattern of morphogenetic events

1. Proximo-distal Gradients

As previously described, most of the cells which migrate out of the primitive streak are destined to give rise to the blood vessels, blood cells and coelomic linings of the yolk sac vascular system. This morphogenesis takes place in a centrifugal pattern in time and space, occurring first in the posterior and posterio-lateral regions of the AOV, then in the anterior and medial parts of the AOV, and finally in the AP itself. As the mesodermal cells become organized into this morphogenetic pattern there is seen to be a gradient of developmental properties which likewise manifests itself in a centripetal direction, from the AOV towards the AP.

This gradient is clearly seen, for example, in the types of morphogenesis of the blood-islands in the AOV and the angioblasts in the AP. As described previously (see Sections 2.3.2 and 2.3.3) when the mesodermal cells enter the AOV some of them clump together to form the large cellular aggregates of the AOV, the blood-islands. These blood-islands then differentiate into the sinusoidal opaca blood vessels containing

large numbers of intravascular blood cells. This is seen to occur when the peripheral cells of the opaca blood-islands flatten and form an endothelial lining over the more central cells of the blood-islands. The developing endothelial cells and the more central blood-island cells subsequently become separated from each other by the formation of extracellular spaces. Meanwhile, the central cells of the blood-islands eventually round up and become free within the vessel lumens as the first developing blood cells. This type of morphogenesis of the yolk sac blood-islands into both blood vessels and blood cells represents the top point in the gradient in a pattern of fading developmental properties. The lower extremity of this gradient is seen in the AP. As outlined previously (Section 2.3.3) in this region the mesodermal cells give rise to the small cellular aggregates of the AP, the angioblasts. The cells of the angioblasts then become arranged to form the endothelial linings of the small pellucida blood capillaries in the absence of the formation of intravascular blood cells. These two extremes in the pattern of vascular morphogenesis are found to run into each other, there being an intermediate zone in the border region between the AOV and AP. In this zone the angioblasts (blood-islands) tend to be larger than those in the rest of the AP, and to give rise to small numbers of intravascular blood cells as well. This observation of a gradient in the sizes and types of morphogenesis of the yolk sac blood-islands and angioblasts is in agreement with the early textbook description of Lillie (1919).

The question then arises as to why the mesodermal cells in the AOV give rise to blood vessels as well as intravascular blood cells whereas those in the AP give rise to blood vessels only. One possibility is that the mesodermal cells in the AOV may be inherently different from those in the AOV, there being a gradient of changing cell populations from the AOV to the AP. An alternative possibility is that the cells in the AOV and AP may be originally identical to each other, but that the environments into which they migrate (i.e., the AOV or AP) may cause them to embark upon the different types of differentiation pathways. This question has been investigated in subsequent chapters of the present thesis (Chapters Three, Four and Six). As will be shown in these chapters, it is found that the environments into which the cells migrate do indeed influence the pathways of differentiation of these cells, the AOV being stimulatory for the differentiation of blood cells

and the AP being unfavourable for the differentiation of blood cells.

A further changing pattern in the type of morphogenesis which occurs in the AOV and AP concerns the methods of coelom formation in these two areas. As described in Sections 2.3.2.2 and 2.3.3.2, it was found that the cellular events involved in coelom formation in the AOV differ from those in the AP. In the AOV the mesodermal cells in the spaces above and between the blood-islands are seen to become arranged to form the walls of small blind channels. These small channels then coalesce with each other to form larger and larger channels. Finally, these coalescing channels unite to form the large coelomic spaces of the AOV. In comparison, in the AP the presumptive coelomic lining cells become arranged to form two adjacent layers of cells dorsal to the angioblastic cords. At first these two layers of cells are very closely apposed to each other. However, clefts soon develop between them, separating the two layers from each other. The resulting space is the extra-embryonic coelom of the AP.

Concomitant with this changing morphogenetic pattern, it was also found that there is a graded pattern in the nature of the coelomic epithelia in the AOV and AP. Thus, whereas the coelomic linings in the AOV were seen to be simple squamous epithelia, the coelomic linings in the AP vary in nature regionally. In the medial part of the area pellucida (associated with, or surrounding, the embryonic axis) the coelomic linings are found to be very thick, being in the form of columnar epithelia. Laterally in the AP the coelomic linings are thinner and form simple squamous epithelia. The simple squamous coelomic linings of the AP extend to the pellucida-opaca junction where they become continuous with the coelomic lining of the AOV.

It is suggested that the reason for these different methods of coelom formation and type of epithelia which are formed in the AP and AOV is the gradient in the sizes of the blood-islands (or angioblastic cords) which are formed in these two areas, and the resultant distribution of the surrounding mesodermal cells in the sheet (compare Figs. [illegible] and [illegible]). As indicated previously, the blood-islands in the AOV tend to be very large and the presumptive coelomic lining cells are distributed in the spaces above and between them. In terms of this observa-

tion, it is significant to note that the formation of the coelomic linings in the AOV involves the participation of small groups of cells within the spaces above and between the blood-islands. These cells therefore form small vesicles in these spaces which later coalesce with each other to form the large coelomic spaces of the AOV. During the formation of these channel linings, the region of contact between adjacent cells is seen to be kept to a minimum. The cells become flattened and widely spread (i.e., squamous cells) with only their narrow lateral surfaces apposed. In the AP, on the other hand, the angioblasts are relatively small and the presumptive coelomic lining cells form a thick continuous sheet dorsal to them. These cells can therefore become organized directly into two continuous cellular layers which finally split apart to form the cavity of the coelom. In these regions the formation of the coelom is therefore seen to involve the participation of larger numbers of more closely packed cells. This is especially apparent in the medial parts of the AP (i.e., the lateral plate mesoderm) in which adjacent coelomic lining cells maintain large areas in contact with each other, giving rise to columnar or cuboidal epithelia.

This explanation for the different types of organization undergone by the presumptive coelomic lining cells of the AOV and AP is an interpretation based on histological observations only, the evidence therefore being circumstantial. An in-depth experimental investigation of the system would be necessary to elucidate the causative factors more reliably.

2. Dorso-ventral Polarity

A further pattern which is seen during the morphogenesis of the yolk sac vascular system is a dorso-ventral polarity which is clearly observable in the spatial relationships of the parts of the system to the overlying ectoderm and the underlying endoderm.

The first visible sign of yolk sac vasculogenesis is the clumping together of the prospective blood-island cells, which occurs in juxtaposition to the yolk sac endoderm. The rest of the mesodermal cells then become organized to form the linings of the extraembryonic coelom dorsal to the blood-islands. In the course of this morphogenesis the ventral lining of the coelom comes to cover the underlying blood-islands

and endoderm, and the dorsal lining of the coelom comes to underlie the ectoderm. The large coelomic space is situated in between. The developing blood vessels are therefore seen to become spatially compartmentalized next to the endoderm, and separated from the ectoderm by the cell-lined coelomic space.

This development of the blood vessels in juxtaposition to the endoderm is in functional accordance with their role in the mobilization of nutrients from the underlying yolk to the developing embryo. The spatial relationship of the developing blood system is also consistent with the findings of Wilt (1965a) and Miura and Wilt (1969) that the yolk sac endoderm exerts a salutary effect on the morphogenesis and erythropoietic differentiation of the blood-islands.

An important finding from the present thesis which is of further relevance to the arrangements of the parts in this dorso-ventral pattern is that the ectoderm is found to exert an inhibitory effect on the erythropoietic differentiation of the blood-islands (see Chapter Four, Section 4.3.2). This inhibitory effect is found to be coupled with the tendency of the tissue, in the presence of the ectoderm but in the absence of the endoderm, to spread out and become flattened in culture (Chapter Four). This could be interpreted to suggest that one of the influences responsible for the creation of this spatial pattern is that the ectoderm provides a substratum conducive to the spreading and flattening of the mesodermal cells (e.g. in the formation of the coelomic linings), whereas the endoderm provides a substratum conducive to the clumping together of the prospective blood-island cells and their subsequent erythropoietic differentiation (see Discussions in Chapters Four and Six).

The above analysis suggests that the structurally and functionally integrated development of the system as a whole is dependent upon the spatially appropriate relationships of the component parts to each other.

A further aspect in the development of this system which occurs in a dorso-ventral pattern concerns the morphogenesis of the central cells of the opaca blood-islands. As described in Section 2.3.2.5, the central cells of the blood-islands are initially closely surrounded by

the developing endothelial cells. However, spaces develop between the developing endothelial cells and the central blood-island cells, separating the two cell-types from each other. As these spaces enlarge and the diameter of the vessels increases, the central blood-island cells are seen to become displaced to the dorsal side of the vessels. During these stages the major portions of the intravascular blood-islands appear to remain attached to the dorsal vessel walls, and they appear to be carried upwards away from the ventral vessel walls. However, some of the ventral-most cells of the intravascular blood-islands tend to maintain their attachments to the ventral endothelial walls, forming tapering ventral trails which span the vessel lumens dorso-ventrally.

During these stages the peripheral cells of the intravascular blood-islands tend to be flattened and very closely applied to the surfaces of the blood-islands. Likewise, the ventral-most cells of the intravascular blood-islands are seen to flatten over the ventral endothelium or to form long narrow cellular ribbons which span the vessel lumen dorso-ventrally. This tendency of the cells of the intravascular blood-islands to be flattened and attenuated appears to be a transient feature in their morphogenesis. During the subsequent stages the cells of the intravascular blood-islands are seen to become progressively more rounded and they finally become liberated into the vessel lumens as the first developing blood cells. However, the dorso-ventral polarity is maintained even in this rounding off of the intravascular blood-island cells. The transformation of the flattened or attenuated intravascular blood-island cells into rounded cells tends to occur first in the dorsal portions of the blood-islands and then later in the tapering ventral portions of the blood-islands. In this temporal sequence, the dorsal blood-island cells and subsequently also the ventral blood-island cells are seen to become liberated into the vessel lumens as the first developing blood cells.

The above observations of the tendency of the cells of the intravascular blood-islands to become flattened and attenuated before they round off is of significance because it indicates that the cells of the blood-islands still possess the ability to become flattened and to form surface linings even when they are already surrounded by an endothelium.

2.4.3.3 Possible explanations for previous discrepancies in the descriptions of blood-island differentiation

As indicated previously (see Sections 1.2.2 and 1.2.3, Chapter One), disparities have existed in the literature with respect to the descriptions of the morphological events involved in yolk sac vasculogenesis. These disparities have stemmed from the descriptions of Sabin (1917, 1920), Sugiyama (1926) and Houser *et al* (1961) which are not in agreement with the currently accepted descriptions of yolk sac vasculogenesis (e.g. Murray, 1932; Edmonds, 1966; Gonzalez-Crussi, 1971; Murphy and Carlson, 1978). In spite of this, a survey of the literature has revealed that the description of Sabin (1920) has traditionally been, and still is, an oft-quoted source of the cellular events involved in yolk sac vasculogenesis (see, for example, Small and Davies, 1972; Zagris, 1980). In view of the above, one of the aims of the present study was to investigate the situation in an attempt to uncover possible reasons for the differences in interpretation provided by the Sabin-Sugiyama-Houser camp on the one hand, and the currently accepted descriptions on the other.

As suggested previously (Section 1.2.3, Chapter One), one of the reasons for the discrepancies appears to stem from the fact that the descriptions of Sabin, Sugiyama and Houser were based on observations in the AP rather than the AOV, but were then used as a basis for describing the morphogenetic events in the whole of the yolk sac. In contrast, the descriptions of the currently accepted authors in the field (e.g. Mato *et al*, 1964; Edmonds, 1966; Gonzalez-Crussi, 1971; Murphy and Carlson, 1978) were based on observations in the AOV itself. The observations in the present study were therefore made in both the AOV and the AP, in an attempt to gain an integrated understanding of the developmental events which occur in both regions.

It has already been mentioned above (Section 2.4.3.2), that the morphogenesis of the yolk sac vascular system appears to involve a fading of developmental properties. Thus, the blood-islands in the AOV are seen to give rise to blood vessels containing large numbers of intravascular blood cells, the blood-islands in the so-called 'intermediate zone' are seen to give rise to blood vessels containing small numbers of intravascular blood cells, and the blood-islands (angioblasts) in

the AP tend to give rise to empty blood vessels only.

As described previously (Section 2.3.2), the present observations in the AOV are essentially in agreement with the currently accepted descriptions in the literature (e.g. Mato *et al*, 1964; Edmonds, 1966; Gonzalez-Crussi, 1971; Murphy and Carlson, 1978). In agreement with these studies, the peripheral cells of the opaca blood-islands are seen to flatten and differentiate into the vessel endothelium while the central cells of the blood-islands differentiate erythropoietically and subsequently become liberated into the vessel lumens as the first developing blood cells.

As also described previously (Section 2.3.3.1), the present observations of the cellular events which occur in the 'intermediate zone' (i.e., in the border region between the AOV and AP) are in agreement with the observations recorded as occurring infrequently in the AP by Sabin (1920), Sugiyama (1926) and Houser *et al* (1961). As described by these authors, the peripheral cells of these blood-islands are seen to flatten and form an endothelium, leaving a small number of central blood-island cells which subsequently give rise to the intravascular blood cells.

However, when the present observations in the AP itself are compared with those of Sabin, Sugiyama and Houser *et al*, it is found that there are large discrepancies between the present and these previous descriptions. As described above (Section 2.3.3.1), it is found in the present study that most of the pellucida blood-islands (angioblasts) give rise to empty blood vessels in the absence of the formation of intravascular blood cells. This is seen to occur when the angioblastic cells become arranged to form an endothelial lining around a central lumen. These blood vessels remain free of intravascular blood cells until the yolk sac circulation becomes functional. Thereafter, the pellucida blood vessels become secondarily filled with blood cells which are transported through the circulation from the vessels of the AOV.

In contrast to this description, Sabin (1917, 1920), Sugiyama (1926) and Houser *et al* (1961) believed that the pellucida angioblasts first became transformed into hollow blood vessels as a result of the complete degeneration of the central angioblastic cells and the organization of

the remaining rim of cells into an endothelium. These authors then described the newly produced vessels as subsequently becoming secondarily filled with blood cells which were described as being produced *de novo* by means of the budding off (by mitosis) of endothelial cells into the lumens of the vessels.

When their descriptions are compared with the present observations it is seen that Sabin, Sugiyama and Houser *et al*, like the present study, noted the initial formation of empty pellucida blood vessels and their subsequent filling up with intravascular blood cells. However, their interpretations of the cellular events involved in these morphogeneses differ from the present interpretation.

In terms of the present study, the formation of the pellucida blood vessels is seen to involve the rearrangement of the angioblastic cells into an endothelium without the degeneration of the central angioblastic cells. In contrast to Sabin (1917, 1920), Sugiyama (1926) and Houser *et al* (1961), no pycnotic nuclei or degenerating vacuolated cells were observed in the present study. It could be suggested that the culturing conditions under which the observations of Sabin, Sugiyama and Houser *et al* were made, may well have been responsible for the cell deaths and degenerations reported by these authors.

The present study also uncovered no evidence for an endothelial origin of the blood cells. Indeed, the pellucida blood vessels were seen to remain free of intravascular blood cells until the circulation commenced and blood cells were mobilized from the vessels of the AOV into those in the AP. It is suggested that the 'newly produced' blood cells described by Sabin (1917, 1920), Sugiyama (1926) and Houser *et al* (1961) probably also originated in the AOV, and entered the pellucida blood vessels via the circulation. It is further suggested that Sabin's, Sugiyama's and Houser's descriptions of the *de novo* production of intravascular blood cells (from the vessel endothelium itself) is an interpretation which would have brought their observations into line with the already present theories of blood-island differentiation. By 1919 it was already commonly known that the yolk sac blood-islands gave rise to both the vessel endothelium and the intravascular blood cells (see, for example, Lillie, 1919). In this regard, it is interesting to note that Sabin and Houser *et al* both cited Lillie's textbook in their bibliographies,

Sabin citing the 1908 edition and Houser the 1930 edition. On the other hand, Sabin's, Sugiyama's and Houser's descriptions were based on observations in the AP and not in the AOV, and indeed, by 1919 it was also already known that the pellucida blood-islands (angioblasts) tend to differentiate into blood vessels in the absence of the formation of blood cells (see Lillie, 1919). It would therefore appear that the discrepancies between the Sabin-Sugiyama-Houser theories and the currently accepted theories are due to the fact that Sabin (1917, 1920), Sugiyama (1926) and Houser *et al* (1961) made their observations in the AP, but then interpreted them in terms of the morphological events which occur in the AOV.

2.4.4 The determination and erythropoietic differentiation of the blood-island cells

As outlined previously, the cells which give rise to the yolk sac vascular system are initially in the form of an irregular sheet of migrating mesenchymal cells. The first morphological sign of yolk sac vasculogenesis is visible when some of the cells in the sheet are seen to clump together against the yolk sac endoderm to form the blood-islands. This is first apparent in the AOV of stage 5-6 blastoderms (head-process to head-fold stage; 22 to 24 hours incubation). These cells subsequently differentiate into the blood vessels and erythropoietic blood cells of the early chick blastoderm. This occurs when the peripheral cells of the blood-islands flatten and form an endothelial lining over the more central cells of the blood-islands. The central cells of the blood-islands meanwhile differentiate erythropoietically, haemoglobin becoming cytochemically detectable by stage 9 (7 somite stage; 34 hours incubation) (O'Brien, 1961; Wilt, 1962, 1965b; Small and Davies, 1972). These cells subsequentiy round off and become liberated into the vessel lumen as the first erythropoietic blood cells.

Although the first visible sign of blood-island formation is apparent at stage 5-6 (22 to 24 hours incubation), it has been found that the determination of the potentiality to form blood cells has already occurred by the time the egg is first laid. Wilt (1974) cut up the blastoderms of newly laid, unincubated eggs into very small pieces, cultured them, and scored their erythropoietic potential. He found that those fragments derived from the horse-shoe shaped region at the posterior end of the

blastoderm were able to differentiate erythropoietically. A similar finding was previously made from explantation experiments carried out on older stages (Settle, 1954; Spratt and Haas, 1961). These experiments showed that at 22-24 hours before the appearance of any morphological or chemical signs of differentiation there is already a definite determination of the potentiality for haemoglobin formation. Indeed, the experiments of Wilt (1974) showed that this commitment has already been made by the time the egg is laid, which is the period approximating the beginning of hypoblast formation (stage XI onwards) (Eyal-Giladi and Kochav, 1976).

Studies on the timing of the synthesis of globin mRNA have shown that most of the globin mRNA has already been synthesized by the time the blood-island cells are first seen to aggregate together in the AOV. Chan *et al* (1974) followed the initial synthesis of globin mRNA in cultured embryonic erythroid cells. These workers used ^{3}H-labelled single-stranded DNA complementary to embryonic chick globin mRNA (globin cDNA) as a probe for detecting and quantifying chick globin mRNA. The results of this study showed that large amounts of globin mRNA were already present in the proerythroblasts by 24 hours of culture (equivalent to the head-fold stage; stage 6 - the stage during which the blood-island cells *in vivo* are clumping together). Thus, the synthesis of globin mRNA must already have begun prior to this stage. When the potential erythropoietic cells of the DPS stage (stage 4; 18 hours incubation - the stage during which the mesodermal cells are migrating from the primitive streak) were tested, only minimal quantities of globin mRNA were detected (Chan *et al*, 1974).

These findings complement the observations of earlier experiments in which chick embryo explants or cultured proerythroblasts were treated with actinomycin D (an inhibitor of RNA synthesis) or 5-bromodeoxyuridine (a thymidine analogue which blocks cell differentiation). The application of these agents at the DPS stage (stage 4; 18 hours incubation) completely blocked the subsequent synthesis of haemoglobin which is normally detectable by the 7 somite stage (stage 9; 34 hours incubation). On the other hand, the application of these agents after the attainment of the head-fold stage (stage 6; 24 hours incubation) permitted the subsequent synthesis of haemoglobin to occur (Wilt, 1965b; Hagopian *et al*, 1972; Chan and Ingram, 1973; Ingram *et al*, 1974).

The results of these experiments, and those of Chan *et al* (1974), therefore indicate that the synthesis of most of the globin mRNA occurs during the period between the DPS- (stage 4) and head-fold (stage 6) stages. This is the period during which the mesodermal cells migrate out from the primitive streak and start to clump together against the yolk sac endoderm. The question which has not been clarified in these studies, however, is whether the synthesis of globin occurs prior to, or as a consequence of, the clumping together of the blood-island cells.

A further finding from the above experiments is that there is a separation of over ten hours between the time of the synthesis of globin mRNA (stage 6; 24 hours incubation) and the first appearance of haemoglobin itself (stage 9; 34 hours incubation). This observation led workers to suggest initially that the globin mRNA molecules were probably stable and long-lived, being transcribed well in advance of their translation (Wilt, 1965b). Suggestions of a post-transcriptional regulation of the expression of the globin genes were also considered (Wilt, 1965b). However, subsequent studies on the transcriptional and post-transcriptional processes involved have shown that the synthesis of globin polypeptides starts soon after the globin mRNA has been transcribed, i.e., at the head-fold stage (stage 6; 24 hours incubation), there being no long time interval separating the two events (Wilt, 1974; Chan, 1976; Hyer and Chan, 1978).

Chan (1976) investigated the synthesis, transport and translation of globin mRNA in embryonic erythroid cells, using ^{3}H-labelled globin cDNA as a probe to quantify and establish the intracellular location of globin mRNA. This worker found that globin mRNA was synthesized and retained exclusively in the nuclei during the initial 24 hours of culture. Thereafter, there was an almost complete transfer of the globin mRNA from the nuclei into the cytoplasm within the next 24 hours. (In terms of our more recent knowledge (see Kinniburgh *et al*, 1978; Tilghman *et al*, 1978a,b; and review by Crick, 1979) the globin mRNA would now have been spliced). During this period the relative amounts of globin mRNA in the nuclei and cytoplasm were seen to change reciprocally, with the total amount of cellular globin mRNA remaining relatively constant throughout the period of erythroid maturation. It was found, furthermore, that haemoglobin became cytochemically detectable only when 75% of the total globin mRNA was present in the cytoplasm. This time period corresponds

to about stage 9 (7 somites; 34 hours of incubation) in the living blastoderm.

Hyer and Chan (1978) then investigated the initial time of synthesis of the globin peptides themselves, using three analytical techniques, viz., SDS-polyacrylamide gel electrophoresis, acid urea polyacrylamide gel electrophoresis, and two-dimensional gel electrophoresis. Their results indicate that globin peptide chains are synthesized at the initial time when globin mRNA first enters the cytoplasm (i.e., at the head-fold stage; stage 6), and that there is no apparent time gap between these two events. This suggests that globin is already being synthesized in the blood-island cells when they first clump together. The question still to be clarified, however, is whether this transport and translation of the globin mRNA occurs prior to, or as a consequence of, the clumping together of the blood-island cells (i.e., is it a cause or a consequence of clumping?).

The findings of the above experiments indicate that there is a delay between the initial synthesis of globin polypeptides (stage 6; 24 hours incubation) and the first appearance of cytologically detectable haemoglobin (stage 9; 34 hours incubation). Hyer and Chan (1978) have suggested two possible explanations for this apparent delay in the appearance of cytologically detectable haemoglobin. One is that haemoglobin may actually be formed as soon as the globin polypeptide chains are synthesized (stage 6), but that the amounts of haemoglobin present prior to stage 9 may be too low to be detected cytochemically by the benzidine stain. This possibility was earlier predicted by Wilt (1974). This worker, using the colorimetric data of Hell (1964) to quantify the amount of haemoglobin per blastoderm at the 7 somite stage (stage 9), and using the estimate of the number of erythropoietic cells as computed by Weintraub *et al* (1971), calculated that there are already about 1,4 x 10^7 molecules of haemoglobin polypeptide per cell by stage 9 (7 somite stage - the stage at which haemoglobin can first be detected). Extrapolating the rate of accumulation of haemoglobin molecules in these cells from the data of Weintraub *et al* (1971), Wilt (1974) estimated that the synthesis of haemoglobin must have begun much earlier than the 7 somite stage, possibly as early as the head-fold stage (stage 6). As suggested by Wilt (1974), "Even if these calculations are in error by more than an order of magnitude, it is clear that there is a considerable

amount of Hb present by the time the most sensitive tests that have been used are applicable." (Wilt, 1974 page 104).

A second possibility suggested by Hyer and Chan (1978) is that haem may not be present in sufficient quantities in the cells prior to stage 9, so that although globin polypeptides are present in the cells as early as stage 6, they do not become incorporated into the final tetrameric form of haemoglobin until some time later. The resolution of these possibilities awaits further experimentation.

Reviewing the findings of these authors in terms of the histological descriptions of yolk sac vasculogenesis (Section 2.3), the following points emerge: As shown by Wilt (1974), the determination of the potentiality for an erythropoietic differentiation has already occurred in the presumptive erythropoietic cells of the chick blastoderm by the time the egg is laid. This is at least 6-7 hours before the beginning of the invagination of the presumptive mesodermal cells through the primitive streak, and 18 hours before the DPS stage (stage 4) (Hamburger and Hamilton, 1951). However, the synthesis of globin mRNA only starts to occur in the period between the DPS stage (stage 4; 18 hours incubation) and the head-fold stage (stage 6; 24 hours incubation). This is the time period during which the mesodermal cells first migrate into the AOV and are seen to clump together against the yolk sac endoderm to form the blood-islands (see Sections 2.3.1 and 2.3.2). As suggested previously, it would be of relevance to determine whether the synthesis of globin mRNA occurs prior to, or as a consequence of, the clumping together of the blood-island cells. A further question in this regard is whether the synthesis of globin mRNA is triggered by the salutary effect (Wilt, 1965a; Miura and Wilt, 1969) of the opaca endoderm on blood-island differentiation.

A further finding from the above studies is that globin synthesis begins soon after the globin mRNA is transcribed and transported from the nucleus into the cytoplasm. Again the question is posed as to whether the transport and translation of the globin mRNA is a cause or a consequence of clumping and/or the salutary effect of the endoderm on blood-island differentiation.

An important point which arises in respect to the histological descriptions of blood-island differen iation concerns the differentiation of the peripheral cells of the opaca blood-islands. As described in the present work, and in agreement with previous descriptions in the literature

(see Section 2.3.2), the peripheral cells of the opaca blood-islands are seen to flatten and give rise to vessel endothelial cells, and not to erythropoietic blood cells. The question then arises as to whether or not these peripheral blood-island cells are also originally synthesizing globin mRNA and globin polypeptides at stage 6. This leads to the further question of whether these peripheral blood-island cells (which will subsequently differentiate into the vessel endothelium) are originally already different from the more central blood-island cells even before blood-island formation, and whether this inherent difference causes them to take on a superficial position in the blood-islands. The alternative hypothesis would be that all the blood-island cells are originally identical in terms of their developmental potentialities, and that the pathways of differentiation upon which they finally embark (into either endothelial cells or blood cells) is dependent upon subsequent environmental factors, e.g., the cells which find themselves on the outside of the blood-islands form endothelium, while the cells which become enclosed in the centre give rise to intravascular blood cells. (This hypothesis was originally put forward by Weiss (1953) and is discussed in more detail in Chapter Six (see Section 6.4)).

Extending the present discussion even further, it would likewise be relevant to determine whether or not the cells of the pellucida angioblasts (which will subsequently give rise to vessel endothelia only, and not to blood cells) are likewise synthesizing (or not synthesizing) globin mRNA and globin during their early stages of differentiation. Again, the question may be posed as to whether or not these cells are originally different from those blood-island cells which will subsequently give rise to blood cells.

An answer to these questions might be obtained using ^{3}H-labelled globin cDNA, or fluorescein-labelled anti-globin antibody, as probes to compare the amounts of globin mRNA, or globin, present in the central and peripheral cells of the opaca blood-islands, and in the cells of the pellucida angioblasts, during successive stages of differentiation.

Further aspects of these questions of whether or not the presumptive endothelial cells are originally different from the presumptive erythropoietic cells, or whether environmental influences play a role, are dealt with in Chapters Three, Four and Six of the present thesis. In these

studies the mesodermal cells have either been heterotopically transplanted from one region of the blastoderm to another (e.g. from the AOV to the AP, and vice versa) or have been cultured in different combinations of the germinal layers. The results of these experiments indicate that environmental influences are indeed an important aspect affecting the pathways of differentiation embarked upon by the mesodermal cells (see Chapters Three, Four and Six).

The findings from the previous studies that haemoglobin is already cytochemically detectable in the blood-island cells at stage 9 (7 somite stage; 34 hours incubation) (O'Brien, 1961; Wilt, 1962, 1965b; Small and Davies, 1972) is also of interest to the present study. As found by Small and Davies (1972), t[illegible] the period prior to the rounding up and subsequent liberation of the intravascular blood-island cells. Indeed, it is found in the present study (Section 2.3.2.5) that this is the period during which some of the intravascular blood-island cells still exhibit the tendency to become flattened and attenuated, and to form cellular linings over surfaces. The question then arises as to whether these flattened and attenuated intravascular blood-island cells are likewise synthesizing haemoglobin during this period, or whether haemoglobin is only synthesized by these cells once they have become rounded. Experiments to answer this question would involve treating histological sections of stage 9 blastoderms with the o-dianisidine stain (for haemoglobin detection) or with fluorescein-labelled anti-globin antibody (for globin detection).

2.4.5 Discussion of the origin and developmental fates of the yolk sac-derived erythropoietic cells and their relationships to the intraembryonic haemopoietic cells

As outlined previously, the first developing blood cells of the chick blastoderm develop from the yolk sac blood-islands in the AOV (see Sections 2.3.2 and 2.3.4). For many years there has been much controversy in the literature as to whether these yolk sac blood-island cells are the cells of origin of all the embryonic haemopoietic cells (see Metcalf and Moore, 1971). The alternative arguments have suggested that the intraembryonic haemopoietic stem cells may originate within the intraembryonic haemopoietic organs themselves (see Rifkind *et al*, 1969) or may originate from some other intraembryonic sources (Dieterlen-Lièvre,

1975, 1978; Martin *et al*, 1978, 1980; Beaupain *et al*, 1979). A discussion of these arguments, and the resolution thereof, follows.

Moore and Owen (1965, 1967a) and Owen and Ritter (1969) used chick embryo parabionts to investigate the question of whether the haemopoietic stem cells were endogenous to the intraembryonic haemopoietic organs, or whether the haemopoietic organs became seeded with extrinsically derived stem cells. Using as markers the sex chromosome differences recognizable between male (ZZ) and female (ZW) mitotic cells, these workers found high degrees of cellular chimaerism in all the haemopoietic organs of the parabionts. This indicated that the haemopoietic organs were indeed populated by blood-borne stem cells. Moore and Owen (1967a) strengthened their argument in experiments in which the haemopoietic organs of irradiated host embryos were shown to become recolonized by blood-borne donor haemopoietic cells, previously isolated from the yolk sac or from the other haemopoietic organs of 7 day donor embryos.

Since the yolk sac is the first site of embryonic haemopoiesis, Moore and Owen (1967b) proposed that the yolk sac was the site of origin of all the haemopoietic stem cells. According to this hypothesis, some of the original blood-island cells do not enter into the primitive- or definitive lines of erythropoietic differentiation, but continue to divide and to give rise to further haemopoietic stem cells. These yolk sac-derived stem cells would later colonize the intraembryonic haemopoietic organs via the circulation (Moore and Owen, 1967b). A yolk sac source of haemopoietic stem cells was also proposed for the mouse embryo (Moore and Metcalf, 1970) from experiments in which injected mouse yolk sac cells were found to repopulate all the haemopoietic organs of irradiated recipient mouse embryos.

This hypothesis, although popular, was not universally accepted (see Harrison and Russell, 1972; Marks and Rifkind, 1972a,b; Dieterlen-Lièvre, 1975, 1978; Martin *et al*, 1978). The main criticisms of the interpretations of Moore and coworkers were:

1. In the parabiosed system used by Moore and Owen (1965), the yolk sacs of the two partners were present, as well as possible intraembryonic sources from the two parabionts. Thus, although chimaerism was clearly demonstrated, there was no proof that the exchanged cells arose from the yolk sac itself.

2. In the irradiation and transplantation work of Moore and Owen (1967a) and Moore and Metcalf (1970), the donor cells were taken from the yolk sacs of 7 day chick embryos and 7-15 day mouse embryos, respectively, i.e., at a time after the circulation had been established, and when there had been ample time for the cells to move from site to site. Thus, there was no proof that the haemopoietic cells which were taken from the yolk sac originated in the yolk sac.
3. The chromosome marker technique used by Moore and Owen (1965, 1967a) gives information about dividing cells only. These workers could thus not rule out the possibility of the participation of the endogenous organ rudiment cells in haemopoiesis. In this regard, an ultrastructural study of foetal hepatic erythropoiesis in the mouse carried out by Rifkind *et al* (1969) implied the development of endogenous hepatic haemopoietic cells from the hepatic mesenchyme itself.

The question of origin of the intraembryonic haemopoietic stem cells therefore still stood open.

More recently Le Douarin and Dieterlen-Lievre and their respective coworkers have reinvestigated this question in an elegant series of experiments, using the chick-quail marker system of Le Douarin (1973a,b). This technique, based on the structural differences between the interphase nuclei of chick and quail cells, has permitted the identification of the majority of cells at any one time.

Le Douarin and coworkers (Le Douarin and Jotereau, 1973, 1975; Le Douarin *et al*, 1975; Houssaint *et al*, 1976) utilized this technique to investigate the relative contributions of intrinsic and extrinsic cells to the haemopoietic populations of the intraembryonic haemopoietic rudiments. This was done by interspecifically grafting the 5-15 day thymus (Le Douarin, 1973), bursa of Fabricius (Le Douarin *et al*, 1975; Houssaint *et al*, 1976) and bone marrow (Le Douarin *et al*, 1975) into the somatopleure of 3 day quail or chick embryos, and thereafter analysing these organs for cellular chimaerism. By this means, Le Douarin and coworkers demonstrated that the entire haemopoietic population of the thymus, bursa and bone marrow was indeed derived from immigrant stem cells. These workers showed, furthermore, that for each organ rudiment there is a critical period during which stem cell influx occurs. Prior to this period, the haemopoietic organ rudiments do not retain the haemo-

poietic stem cells, even though they are already present in the blood stream (Le Douarin and Jotereau, 1975; Houssaint , 1976).

Having confirmed the proposal of Moore and Owen (1965, 1967a,b) that the haemopoietic organ rudiments are indeed seeded from extrinsic sources, the si e(s) of origin of the haemopoietic stem cells became a focal point. The question still stood open as to whether the circulating haemopoietic stem cells originated in the yolk sac blood-islands (as proposed by Moore and Owen, 1967b, or whether they originated from some other sources.

Dieterlen-Lievre, Martin and coworkers focused attention on this problem. These workers grafted the AP of 2 day quail blastoderms onto the AOV of chick blastoderms of the same stage, and followed the development of the haemopoietic system by histological- and immune haemolysis techniques (Dieterlen-Lièvre, 1975, 19 8; Martin , 1978). Similar experiments were subsequently also carried out with histocompatible chick-chick yolk sac-embryo chimaeras, using sex chromosome markers (Lassila , 1978; Martin , 1979) and differences in IgG allotypes (Martin , 1979) to ascertain the relative contributions of the embryo and yolk sac to the lymphopoietic cell populations. The results of these studies indicated that the haemopoietic organs of the chimaeras were predominantly populated by embryo-derived haemopoietic cells. Furthermore, when Martin : (1978) examined the yolk sacs of the chimaeras, they found that there was an increased recruitment, with age, of embryo-derived haemopoietic cells in the yolk sac itself. Indeed, from day 7 nwards the composition of the yolk sac blood-islands changed from being entirely chick to a mixture of both chick and quail cells (Martin , 1978; see also Dieterlen-Lièvre, 1978). This reverse seeding of the yolk sac by intraembryonic haemopoietic stem cells is a phenomenon not previously recognized.

In terms of these observations, it appears that the yolk sac-derived haemopoietic stem cells which originate from the original yolk sac blood-islands function in haemopoiesis for a limited period only. From day 7 onwards the yolk sac becomes colonized by haemopoietic stem cells which are produced in the body of the embryo itself.

This latter finding provided a clear explanation for the results of the irradiation-reconstitution experiments of Moore and Owen (1967a),

as subsequently repeated and confirmed by Lassila *et al* (1978, 1980), that the 7 day yolk sac contains stem cells capable of replenishing the lymphopoietic organs and giving rise to functional lymphocytes. In terms of the findings of Martin *et al* (1978) it would appear that the lymphoid stem cells present in the 7 day yolk sac are probably secondary immigrants originating in the body of the embryo itself (see Lassila *et al*, 1980).

Attention was then focused on the sources of origin of these intraembryonically derived haemopoietic stem cells. Lassila *et al* (1980) showed that the 7 day intraembryonic mesenchyme, when transplanted into 14 day irradiated chick embryos, was capable of populating the haemopoietic organs and giving rise to functional lymphocytes. Martin *et al* (1980) then attempted to locate the site(s) of these stem cells within the intraembryonic mesenchyme. These workers performed reciprocal chick-quail grafts of the anterior part of the area pellucida of day 2 blastoderms, and thereafter analysed the cellular composition of the haemopoietic organs for chimaerism. The results of this study indicated that the haemopoietic stem cells arise from all parts of the AP excluding the prospective head-neck region. Taking into account the relative positions of the various haemopoietic organs with respect to the chick-quail tissue boundaries, these authors found that each intraembryonic haemopoietic organ rudiment appears to be colonized by precursor cells derived from the adjacent mesenchyme. These findings suggest that the intraembryonic haemopoietic tissues are colonized by the nearest available stem cells rather than by cells arising from a distant source (Martin *et al*, 1980).

Summarizing the above findings, it is now clear that the intraembryonic mesenchyme provides the stem cells for all the haemopoietic organs, and even colonizes the yolk sac blood-islands themselves. In view of these observations, attention has been focused on the yolk sac blood-islands (see Beaupain *et al*, 1979), the question being asked of whether the original yolk sac blood-islands are the sole source of origin of all the primitive- and definitive erythrocytes, or whether the intraembryonic mesenchyme also provides a source.

As outlined previously (see Section 2.3.4.1), the primitive- and definitive series of erythrocytes are two morphologically distinguish-

able erythroid cell types (Lucas and Jamroz, 1961; Edmonds, 1966; Small and Davies, 1972) with electrophoretically distinct haemoglobins (Bruns and Ingram, 1973a,b; Brown and Ingram, 1974; Mahoney *et al*, 1977) whi appear in sequence in the embryonic circulation. From the middl of day 1 to th. end of day 5, only the primitive series of erythrocytes are found in the circulation. These cells develop as a cohort from the original yolk sac blood-islands (see Section 2.3.4.2), reaching a maximum in cell number at eight days of incubation, and then decreasing in number until they are no longer detectable in the circulation by 16 days of incubation (see Lucas and Jamroz, 1961; Bruns and Ingram, 1973a). The definitive series of erythrocytes, stem cell derived, is a sequestered population of cells which makes its first appearance in the embryonic circulation at the end of day 5 (Bruns and Ingram, 1973a). Over the next 10-11 days, the definitive cells increase in proportion, from 1-4% of the circulating erythroid elements at the end of day 5, to 50% at day 7, to 100% of the erythroid line by day 16 (see Bruns and Ingram, 1973a).

In view of the finding of Martin *et al* (1978) that the yolk sac blood-islands become recolonized with embryo-derive stem cells from day 7 onwards, it was tempting to speculate that from day 7 onwards the sequestered population of definitive erythrocytes might likewise be derived from an intraembryonic source.

Hagopian and Ingram (1971) had previously shown that the de-embryonated stage 4 (18 hours incubation) chick yolk sa cultured *in vitro* was capable of producing both the primitive and definitive series of erythrocytes. However, the yield of erythrocytes produced by the explants was found to be very low, constituting only 2,5% of the normal number of erythrocytes on day 8 of incubation. Beaupain *et al* (1979) reinvestigated this question, using chick-quail yolk sac-embryo chimaeras prepared from stage 8-9 blastoderms (30 hours incubation). These authors found that the original yolk sac blood-islands were indeed capable of giving rise to both the primitive- and definitive series of erythrocytes, but that embryo-derived erythrocytes of the definitive line appeared in increasing numbers from days 5 (or 7) to 13. The observations of these authors indicate that the original yolk sac blood-islands give rise to most of the primitive erythrocytes and only a small proportion of the definitive erythrocytes of the early chick blastoderm. On the other

hand, the majority of the definitive erythrocytes originate from within the body of the embryo itself (Beaupain et al., 1979).

It thus appears that up to days 5-7 of incubation the original yolk sac blood-islands are the main (or even sole) source of the circulating primitive- and definitive erythrocytes. From day 5-7, when the yolk sac blood-islands become seeded with stem cells derived from intraembryonic sources, the subsequent populations of definitive erythrocytes are found to be composed mainly of embryo-derived cells (Beaupain et al., 1979).

In terms of these findings, the developing blood cells observed in the present study (Section 2.3.4.2) would probably all have been derived from the original yolk sac blood-islands.

It should be noted that although the yolk sac blood-islands tend to give rise mainly to the primitive erythrocytes and to a small proportion of the definitive erythrocytes (Beaupain et al., 1979), it has nevertheless also been found from the chick-quail yolk sac-embryo chimaera experiments of Martin et al. (1978) that in exceptional cases the yolk sac-derived haemopoietic cells may themselves enter the lymphoid organs of the developing embryo, and differentiate along lymphopoietic lines. Furthermore, Jotereau and Houssaint (1977), investigating the question of the pluripotency of the yolk sac-derived haemopoietic cells, found that when the AOV of the head-fold stage quail blastoderm was associated with the chick precolonization thymic rudiment for 9 days in the somatopleure of a chick host, the developing chick thymus contained quail lymphoid cells. This indicates, therefore, that the yolk sac mesodermal cells from the very earliest stages (the head-fold stage being the stage at which the blood-island cells aggregate together - see Section 2.3.2) are capable of differentiating along lymphopoietic lines when placed in a suitable microenvironment.

Further aspects of the question of the pluripotency of the yolk sac mesodermal cells and the effects of microenvironmental factors on their pathways of differentiation are dealt with in Chapters Three, Four, Five and Six of the present thesis.

CHAPTER THREE

AN INVESTIGATION OF THE EFFECTS OF THE PELLUCIDA AND OPACA ENVIRONMENTS ON THE PATHWAYS OF DIFFERENTIATION OF THE MESODERMAL CELLS, BY MEANS OF THE TRANSPLANTATION OF MESODERMAL FRAGMENTS FROM ONE REGION OF THE BLASTODERM INTO ANOTHER

3.1 Introduction

One of the findings from the microscopical study of the previous chapter (Chapter Two) is that the pathways of differentiation of the mesodermal cells in the AOV tend to differ from those in the AP. Thus, whereas the blood-islands in the AOV are seen to give rise to endothelial vessels containing large numbers of blood cells, those in the AP tend to give rise to empty endothelial vessels only (i.e., vessels in the absence of blood cells). These conditions are found to grade into each other. Furthermore, whereas the coelomic linings in the AOV are seen to be in the form of simple squamous epithelia, those in the medial parts of the AP tend to be in the form of columnar or cuboidal epithelia. These conditions are likewise found to grade into each other (see Chapter Two).

Findings of this nature lead one to question whether the mesodermal cells in the AOV and AP are inherently different from each other, or whether their pathways of differentiation are dependent upon environmental factors inherent in the different regions of the blastoderm (i.e., the AP or AOV). This question was tackled in the present chapter. In these experiments, radioactively labelled mesodermal fragments were isolated from the AP or AOV of donor blastoderms and heterotopically transplanted into the AP or AOV of non-radioactive host blastoderms. After an overnight culturing period, the developmental fates of the transplanted cells were ascertained autoradiographically.

As will be shown in the present chapter, it is found that whereas some of the heterotopically transplanted pellucida and opaca mesodermal cells differentiate in accordance with their original fates, others differentiate in accordance with their new environments. Thus a clear-cut "yes" or "no" answer cannot be given to the question of whether the

pellucida and opaca mesodermal cells are different from each other. Rather, it appears that at the time of transplantation the developmental fates of some of the mesodermal cells have already been fixed, whereas others are still developmentally plastic. The details of these experiments are outlined below.

3.2 Material and Methods

3.2.1 Sterile techniques

All tissue transplantation and culturing procedures were carried out under sterile conditions in front of a lamina flow (John Bass Ltd., Southampton).

All glassware and plastic-ware was first rinsed in running tap water, then soaked overnight in 2% Extran (Merck), washed in ten changes of tap water followed by three changes of double glass-distilled water, and dried in a dust-free drying oven. The glassware was sterilized in a model HA-3D Autoclave (Hirayama Manufacturing Corporation, Japan) for twenty five minutes at $122^{o}C$ and $1,25Kg/cm^{2}$, followed by a thirty five minute drying cycle. The plastic-ware and implements which could not withstand high temperatures were sterilized in 70% ethanol and allowed to air dry in front of the lamina flow.

The dissection instruments (all stainless-steel) were rinsed in running tap water, boiled in 2% Extran (Merck) for fifteen minutes, and washed in ten changes of tap water followed by three changes of double glass-distilled water. The dissection instruments were then sterilized in boiling glass-distilled water for thirty minutes.

Solutions were sterilized by autoclaving for twenty five minutes at $122^{o}C$ and $1,25Kg/cm^{2}$.

Prior to incubation the eggs were washed in running tap water, dried, and wiped down with cottonwool slightly moistened with 70% ethanol.

The incubator was routinely sterilized by wiping down with 70% ethanol, and the ethanol allowed to evaporate totally.

Prior to, and after, each working period, the bench surface in front of the lamina flow was washed and dried, and wiped down with 70%

ethanol.

3.2.2 Tissue transplantation experiments

The tissue transplantation experiments were carried out under sterile conditions, as described above. The procedure involved the isolation of mesodermal fragments from donor blastoderms and their transplantation into known sites in explanted host blastoderms. The host blastoderms were then cultured overnight on solid organ culture medium. The donor cells were radioactively labelled so that their fate could be followed by autoradiography. The procedure is outlined below.

3.2.2.1 Explantation of the host blastoderms onto solid organ culture medium

The host blastoderms were explanted onto solid organ culture medium according to the method of New (1955). This method involves culturing the blastoderms on their vitelline membranes which are stretched across a glass ring.

Chick blastoderms of the required incubation stages (see Experiments and Results, Section 3.3) were dissected from the yolk on their vitelline membranes and transported under saline to a petri dish of saline. The vitelline membrane was arranged so that the blastoderm was uppermost, and a glass ring was positioned on the surface of the vitelline membrane so that it surrounded the blastoderm. The free edges of the vitelline membrane were folded inwards over the ring so that the vitelline membrane formed a flat, unwrinkled surface within the ring. Excess saline was then removed from the petri dish.

The ring with its attached vitelline membrane bearing the blastoderm was transported onto the surface of solid organ culture medium in a watch glass (Section 3.2.2.2). Saline was pipetted into the interior of the ring in preparation for the receipt and implantation of the radioactively labelled mesodermal fragments from the donor blastoderm (Section 3.2.2.3).

3.2.2.2 Preparation of the solid organ culture medium

One part glucose-agar (2% purified Difco agar, 1% dextrose, in chick

embryo saline), previously autoclaved and allowed to cool to 45°C, was added to one part thin albumen at 45°C. The organ culture medium was poured into sterile watch-glasses in petri dishes and allowed to solidify and cool in front of a lamina flow

3.2.2.3 Preparation of radioactively labelled donor tissue for transplantation

Isotopes were administered to the eggs of donor blastoderms prior to their incubation. A 1-2mm hole was made in the shell at the blunt end of the egg, through which 0,1ml of chick embryo saline containing 5-10uCi methyl-^{3}H-thymidine (Radiochemical Centre, Amersham) was injected into the air sac. The hole was sealed with cellotape and the eggs were incubated at 38°C until the required stage of development was reached (see Experiments and Results, Section 3.3).

Radioactively labelled chick blastoderms of the required incubation stages were dissected from the yolk under chick embryo saline and removed from their vitelline membranes. The isolated blastoderms were transported under saline to a petri dish of fresh saline on a stage of a binocular microscope (American Optical Corporation, model 570) for further operational procedures.

Sharp stainless-steel insect pins (D.F. Tayler and Co., England, size 0) mounted on pencil-length glass rods were used for peeling the endoderm from the underlying mesoderm and ectoderm. A hair-loop made with fine infants' hair (Rugh, 1962 page 8) was then used for gently scraping the mesoderm off the ectoderm.

The mesodermal fragments were cut into 50-100µm diameter fragments by working tips of the insect pins against each other in a shearing motion. The mesodermal fragments were then transported onto the surface of the host blastoderm within the glass ring on the surface of the organ culture medium, by means of a micro-syringe with a vernier control (Burroughs Wellcome and Company, England; and G.K.N. Shardlow Metrology Ltd., England).

The stainless-steel insect pins were used to implant the donor tissue fragments into given regions of the host blastoderm (see Section 3.3). This was done by making small slits in the endodermal layer of

the host, through which the donor tissue was inserted into the mesodermal layer of the host blastoderm. The endoderm of the host was then flattened so that it enclosed the donor tissue. At least 15-20 radioactively labelled mesodermal fragements were transplanted thus into each host blastoderm. Thereafter the saline was pipetted from the interior of the ring, care being taken to avoid interfering with the transplants.

3.2.2.4 Culturing of the host blastoderms

The host blastoderms, explanted on solid organ culture medium and implanted with donor tissue as above, were cultured overnight at 38 C. The atmosphere within the petri dishes was kept humid by means of moist tonwool which was placed around the watch-glass in each petri dish.

2.5 Processing of the host blastoderms for autoradiography

After an overnight culturing period at 38°C, the host blastoderms, still attached to their glass rings, were lifted off the surface of the organ culture medium and placed into a petri dish containing Carnoys Fixative A (Humason, 1967), made up of 3:1 by volume of absolute ethanol and glacial acetic acid. The blastoderms were fixed as such for 45 minutes. The blastoderms were then excised from the rings and transferred to absolute ethanol, cleared in methyl benzoate, and embedded in Paraplast (Sherwood Medical Industries, St. Louis, Missouri).

The blastoderms were serial sectioned at 7µm on a Spencer Microtome (model 820), and mounted without adhesive on glass slides, precleaned as follows: The slides were immersed overnight in 2N HCl, washed in ten changes of tap water followed by three changes of double glass-distilled water, rinsed in 95% ethanol and dried with a lint-free cloth. The mounted sections were allowed to dry in a dust-free box.

The Paraplast was removed from the sections with xylol, and the sections were then hydrated through a graded series of ethanol, ending with three changes of double glass-distilled water. The sections were again allowed to dry in a dust-free box.

All autoradiographic work was carried out in an autoradiography dark room using an Ilford 902S safelight filter.

Ilford G5 Nuclear Emulsion was warmed in a 45°C water-bath and diluted 1:2 by volume with 2% glycerol (in deionized water) at 45°C. The slides were individually coated by carefully dipping them in emulsion for three seconds, and slowly withdrawing them. Excess emulsion was removed from the lower edge of the slides by touching them briefly on absorbent paper. The dipped slides were maintained in a vertical position until the emulsion was dry. Thereafter they were placed into clean, dust-free plastic slide-boxes and maintained in the dark at 4°C for twenty one days.

The autoradiographs were developed for three minutes in D19 Developer (0,2% metol; 9% sodium sulphite; 0,8% hydroquinone; 5,2% sodium carbonate; 0,5% potassium bromide; in distilled water) diluted 1:1 with distilled water just prior to use. The slides were dipped in 1% acetic acid for ten seconds, fixed in 25% sodium thiosulphate for six minutes, and washed in running tap water for thirty minutes.

The sections were stained with filtered Mayer's Haematoxylin (Humason, 1967) for five minutes, dehydrated through a graded series of ethanol, cleared in xylol, and coverslips were applied with DPX mountant.

The appropriate controls for autoradiography, as detailed by Rogers (1969), were also carried out.

3.3 Experiments and Results

As indicated previously (Section 3.1), the aim of the present study was to investigate whether the pellucida and opaca mesodermal cells are different from each other, or whether their pathways of differentiation are dependent upon their locations within the blastoderm (i.e., the AP or the AOV). This investigation was carried out by heterotopically transplanting radioactively labelled mesodermal fragments from the AP or AOV of donor blastoderms into the AP or AOV of non-radioactive host blastoderms, and thereafter following the fates of the labelled cells autoradiographically. In the first experiment (Experiment 3.I) the effect of the opaca environment on the pathways of differentiation of the pellucida mesodermal cells was investigated. In the second experiment (Experiment 3.II) the effect of the pellucida environment on the pathways of differentiation of the opaca mesodermal cells was investigated. The

details of these experiments follow below.

3.3.1 Experiments to investigate the effects of the pellucida and opaca environments on the pathways of differentiation of the mesodermal cells

Experiment 3.I : (AP mesoderm transplanted into the AOV)

Radioactively labelled mesodermal fragments were isolated from the AP of stage 8 donor blastoderms and were transplanted into the AOV of non-radioactive stage 5 host blastoderms (Fig. 3.1). These stages were chosen because the mesodermal cells in the areas concerned (stage 8 AP; stage 5 AOV) were still at the very early stages of their overt differentiation (see Chapter Two). This would thus allow for the simultaneous differentiation of the host and donor cells.

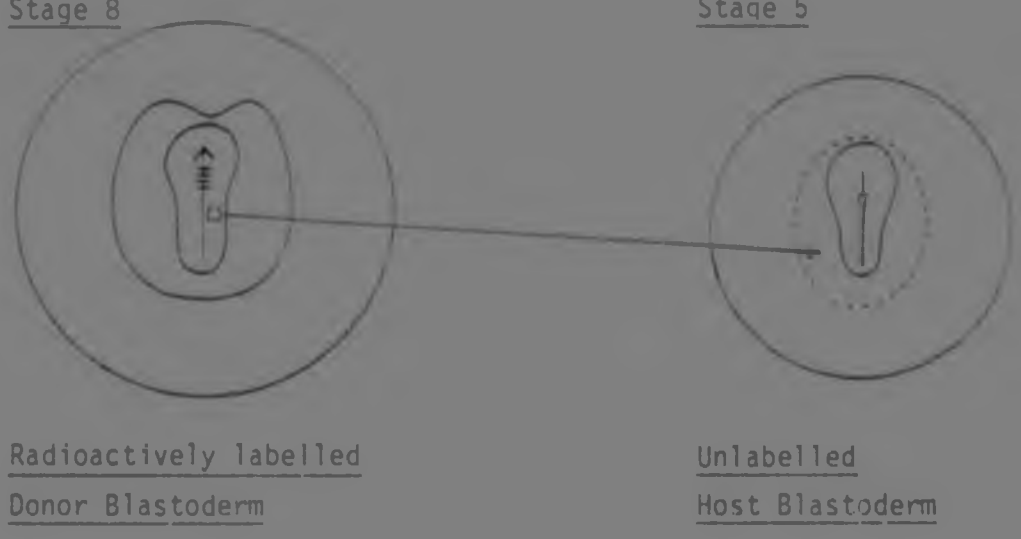


Figure 3.1

Diagram illustrating the transplantation of radioactively labelled mesodermal fragments from the AP of stage 8 donor blastoderms into the AOV of non-radioactive stage 5 host blastoderms.

(Micrographs of the autoradiographic histological sections are presented in Figures 3.2 and 3.3 (pages 150 and 151)).

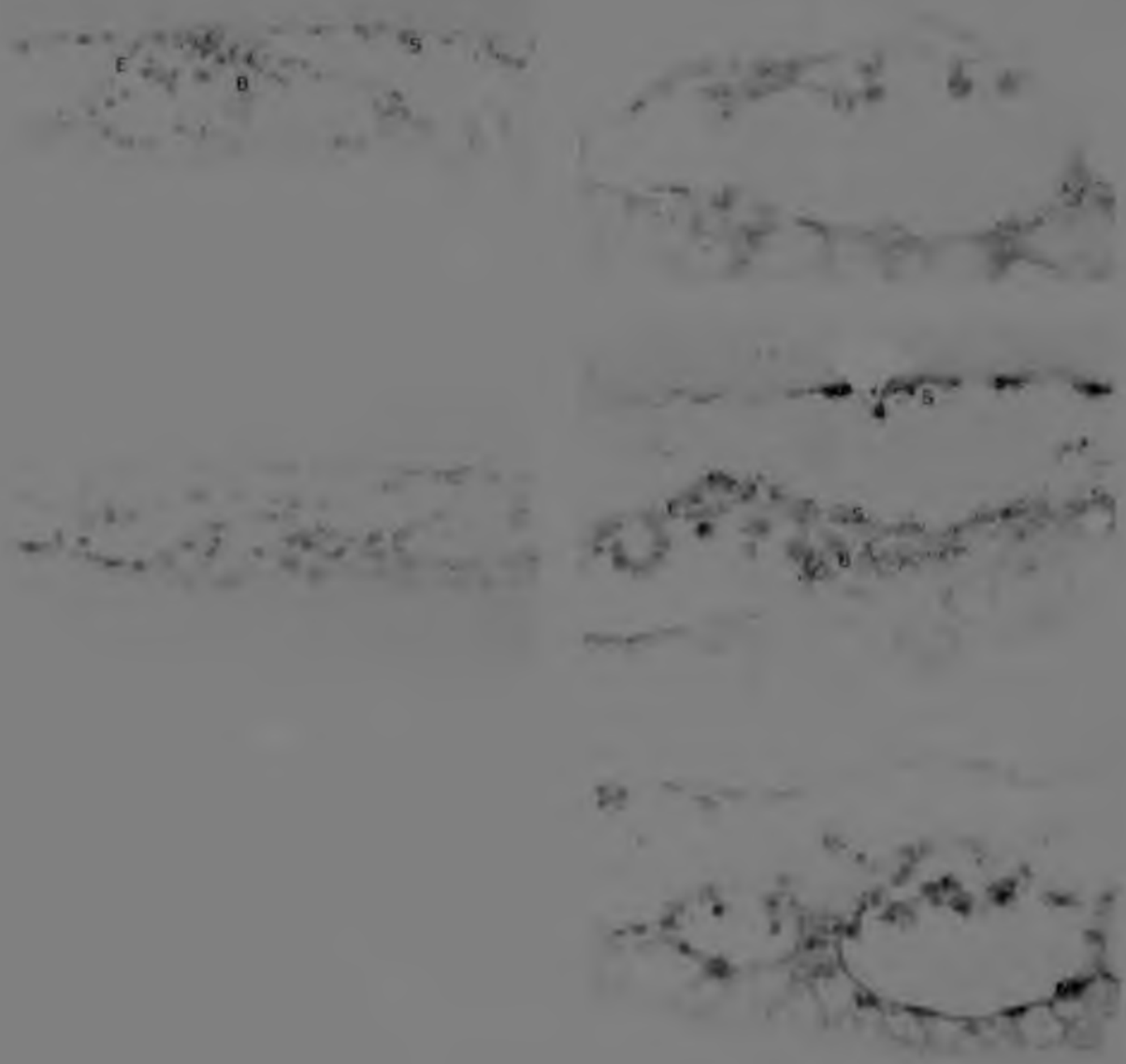


Figure 3.2

Autoradiographic histological sections from Experiment 3.I in which radioactively labelled stage 8 pellucida mesodermal fragments were transplanted into the AOV of non-radioactive stage 5 host blastoderms. Some of the radioactively labelled pellucida mesodermal cells appear to have differentiated in accordance with their original fates, giving rise to dorsal and ventral endothelial cells (E) and dorsal and ventral coelomic lining cells (S). Other radioactively labelled pellucida mesodermal cells appear to have differentiated in conformity with their new environment, giving rise to intravascular blood cells (B) in the AOV of the host.

B, intravascular blood cells; E, endothelial cells;
S, coelomic lining cells

Micrographs on left x 356
Micrographs on right x 711

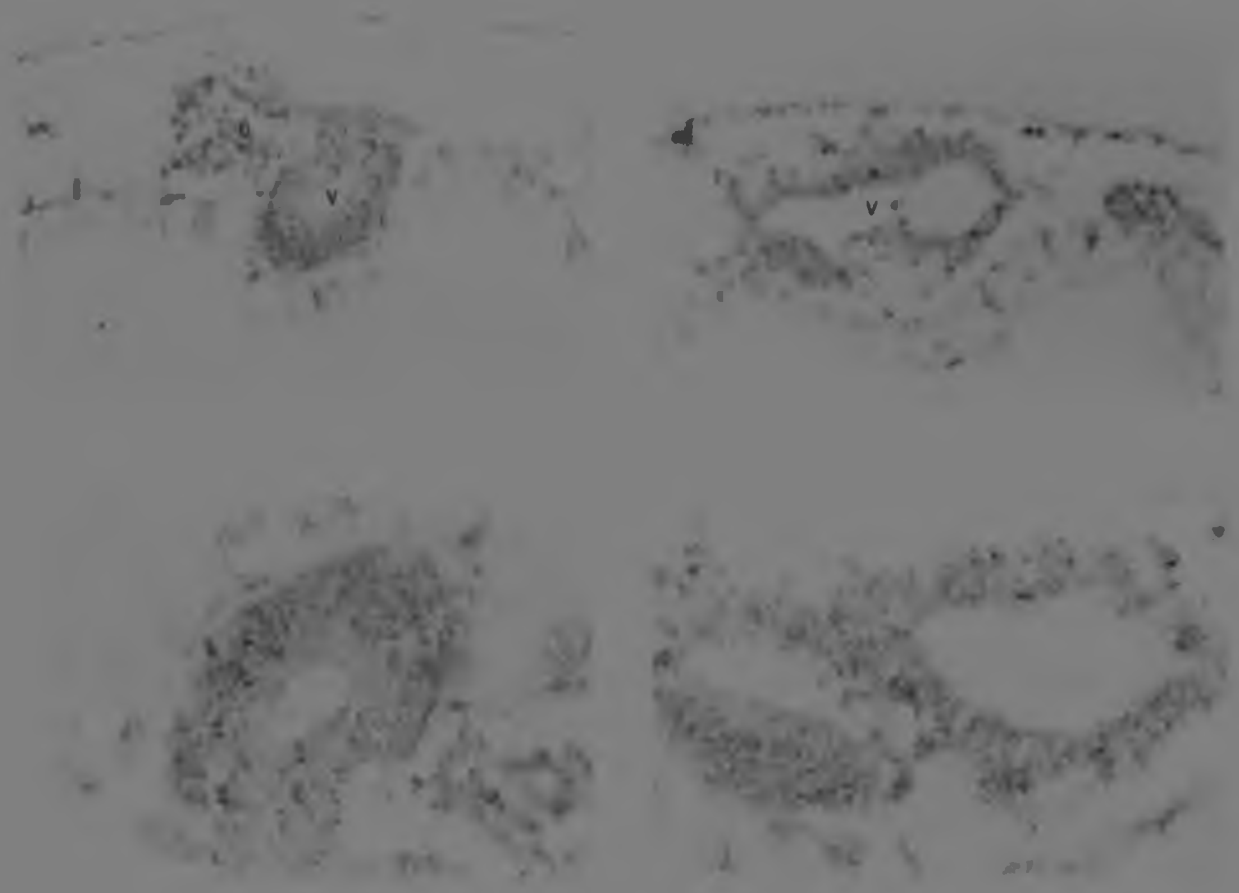


Figure 3.3

Autoradiographic histological sections from Experiment 3.I in which radioactively labelled stage 8 pellucida mesodermal fragments were transplanted into the AOV of non-radioactive stage 5 host blastoderms. In the present micrographs the labelled cells (i.e., transplanted pellucida mesodermal cells) are seen to have formed the walls of thick-walled vesicles 'V). These thick-walled vesicles are reminiscent of the thick-walled coelomic linings which normally develop from the lateral plate mesoderm in the medial parts of the AP.

B, blood cells; V, thick-walled vesicles.

Top micrographs x 356
Bottom micrographs x 711

An examination of the autoradiographic histological serial sections (Figs. 3.2 and 3.3) revealed that some of the transplanted pellucida mesodermal cells appeared to have differentiated in accordance with their original fates, whereas others appeared to have differentiated in conformity with their new environment.

Cases in which the transplanted pellucida mesodermal cells appeared to have differentiated in accordance with their original fates are:

Some of the transplanted pellucida mesodermal cells were seen to have given rise to dorsal and ventral endothelial cells, and dorsal and ventral coelomic lining cells in the AOV of the hosts (Fig. 3.2). These types of linings would also have been formed by the pellucida mesodermal cells if they had remained in the AP.

Some of the transplanted pellucida mesodermal cells were seen to have given rise to the walls of thick-walled vesicles in the AOV of the hosts. These thick-walled vesicles are reminiscent of the thick-walled coelomic linings which normally develop from the lateral plate mesoderm in the medial parts of the AP (Fig. 3.3). Thick-walled coelomic linings are not normally formed in the AOV.

Cases in which the transplanted pellucida mesodermal cells appeared to have differentiated in conformity with their new environment are:

Some of the transplanted pellucida mesodermal cells gave rise to intravascular blood cells in the AOV of the hosts. During normal development the pellucida mesodermal cells do not give rise to blood cells (see Chapter Two).

Experiment 3.II : (AOV mesoderm transplanted into the AP)

Radioactively labelled mesodermal fragments were isolated from the AOV of stage 4-5 donor blastoderms and were transplanted into the AP of non-radioactive stage 8 host blastoderms (Fig. 3.4).

An examination of the autoradiographic histological serial sections revealed that a large number of the transplanted opaca mesodermal cells had moved from the AP into the AOV of the hosts (Fig. 3.5, top two micrographs). Furthermore, these opaca mesodermal cells which had moved into the AOV of the hosts were seen to have differentiated along their normal

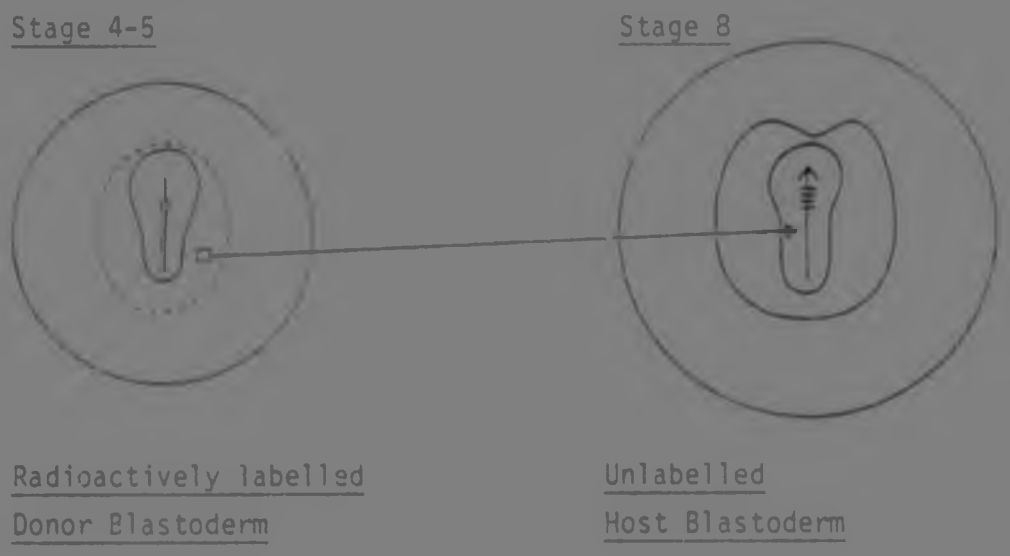


Figure 3.4

Diagram illustrating the transplantation of radioactively labelled mesodermal fragments from the AOV of stage 4-5 donor blastoderms into the AP of non-radioactive stage 8 host blastoderms.

pathways of development, giving rise to coelomic linings, endothelial linings, and blood cell (Fig. 3.5, top two micrographs). On the other hand, of the transplanted opaca mesodermal cells which remained in the AP of the hosts, some of the cells appeared to have differentiated according to their original fates whereas others appeared to have differentiated in conformity with their new environment (Fig. 3.5, bottom two micrographs).

Cases in which the transplanted opaca mesodermal cells appeared to have differentiated in accordance with their original fates are:

Large numbers of labelled intravascular blood cells were found in the AP of the hosts (Fig. 3.5, bottom left micrograph). Large numbers of blood cells are not normally formed in the AP (see Chapter Two).

Cases in which the transplanted opaca mesodermal cells appeared to have differentiated in conformity with their new environment are:

A small number of labelled cells were found in the endodermal

(Text continues on page 155)

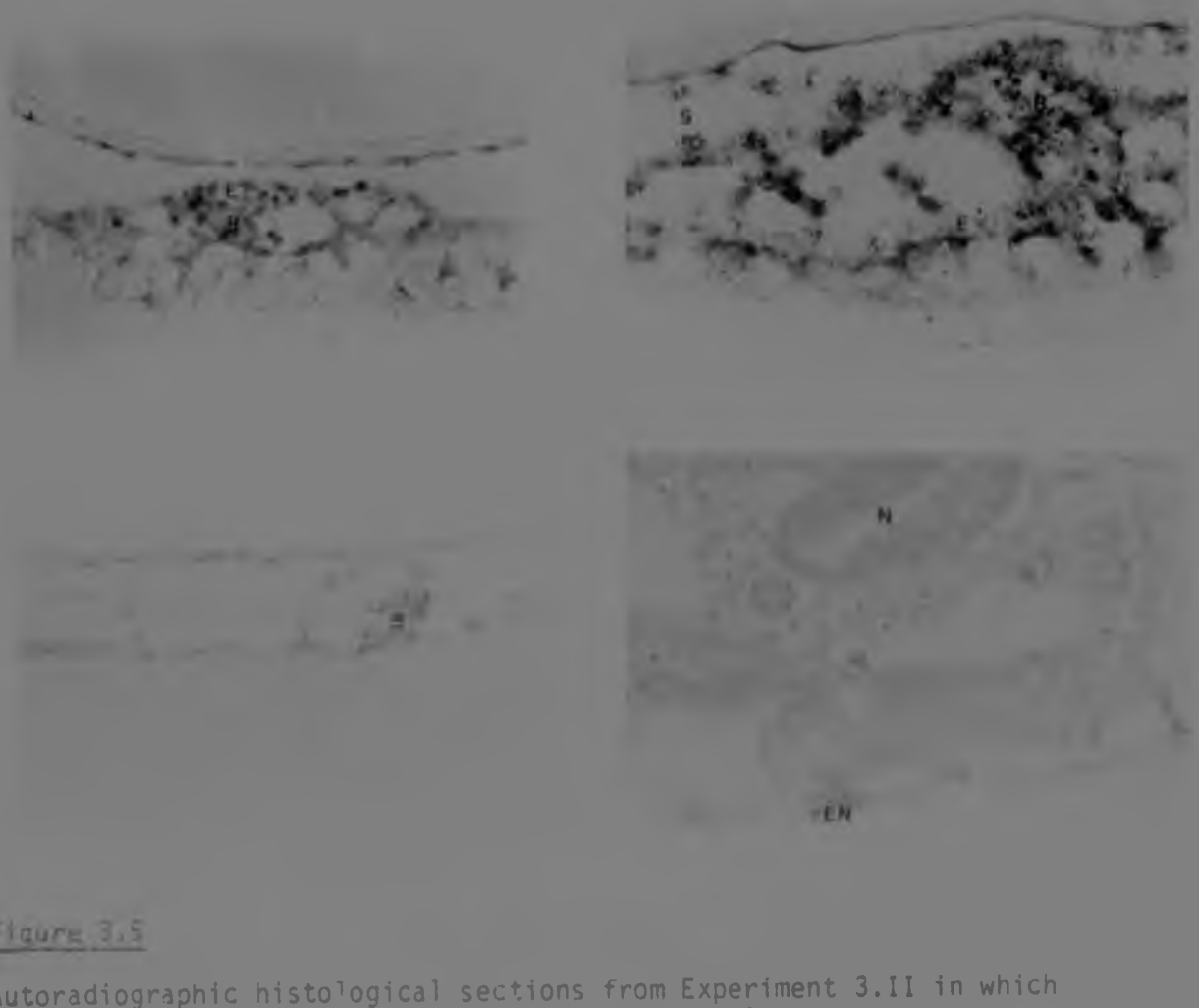


Figure 3.5

Autoradiographic histological sections from Experiment 3.II in which radioactively labelled stage 4-5 opaca mesodermal fragments were transplanted into the P of non-radioactive stage 8 host blastoderms. A large number of the transplanted opaca mesodermal cells are seen to have moved out of the AP into the AOV of the hosts (top two micrographs). These opaca mesodermal cells which have moved into the AOV of the hosts are seen to have differentiated along their normal pathways of development, giving rise to intravascular blood cells (B), endothelial cells (E), and coelomic lining cells (S) (top two micrographs). The remaining transplanted opaca mesodermal cells are seen to have remained in the AP of the hosts (bottom two micrographs). Of these cells, some appear to have differentiated according to their original fates, giving rise to large numbers of intravascular blood cells (B) in the AP of the hosts (bottom left micrograph), whereas others appear to have differentiated in conformity with their new environment, giving rise to endodermal cells (EN) in the AP of the hosts (bottom right micrograph).

B, intravascular blood cells; E, endothelial cells;
EN, endodermal cells; N, neural tube; S, coelomic lining cells.

Top left x 480; Top right x 711
Bottom micrographs, x 356

layer of the AP of the hosts (Fig. 3.5, bottom right micrograph). The mesodermal cells of the AOV do not normally give rise to endodermal cells (see Chapter Two).

3.3.2 Control experiments

In order to ensure that the transplantation procedures were not themselves affecting the types of differentiation of the mesodermal cells, two control experiments (Experiments 3.III and 3.IV) were carried out. In these control experiments, mesodermal fragments were isolated from a given region of the donor blastoderms (either the AP or AOV) and transplanted back into the same region of a host blastoderm of the same developmental stage. After an overnight culturing period, the host blastoderms were processed for autoradiography, and the fates of the transplanted cells were ascertained autoradiographically.

As is described below, it is found that the transplanted cells in these control experiments were able to become incorporated, apparently normally, into the tissues of the hosts and to differentiate together with the hosts' cells. Thus, the transplantation procedures themselves appear to have no overt effect on the differentiation pathways embarked upon by the mesodermal cells. The details of these control experiments follow below.

Experiment 3.III : (AOV mesoderm transplanted into the AOV) (Control)

Radioactively labelled mesodermal fragments were isolated from the AOV of stage 4-5 donor blastoderms and were transplanted into the AOV of non-radioactive host blastoderms of the same developmental stage (Fig. 3.6).

An examination of the autoradiographic histological serial sections revealed that the transplanted cells gave rise to all the normal mesodermal cell types characteristic of the AOV. As shown in Fig. 3.7, the transplanted cells, together with the host cells, were seen to have given rise to intravascular blood cells, endothelial cells, and coelomic lining cells. Both labelled (i.e., donor) and unlabelled (i.e., host) cells contributed to the above structures, indicating that the donor cells



Figure 3.6

Diagram illustrating the transplantation of radioactively labelled mesodermal fragments from the AOV of stage 4-5 donor blastoderms into the AOV of non-radioactive host blastoderms of the same stage.

were able to become incorporated into the tissues of the host and to differentiate together with the host cells. Thus, the transplantation procedures appeared to have no overt effect on the normal differentiation pathways of the opaca mesodermal cells. (See Fig. 3.7, page 157).

The same type of finding was made in the next control experiment in which the mesodermal cells from the AP were transplanted back into the AP.

Experiment 3.IV : (AP mesoderm transplanted into the AP) (Control)

Radioactively labelled mesodermal fragments were isolated from the AP of stage 8 donor blastoderms and were transplanted into the AP of non-radioactive host blastoderms of the same developmental stage (Fig. 3.8).

(Text continues on page 158)



Figure 3.6

Diagram illustrating the transplantation of radioactively labelled mesodermal fragments from the AOV of stage 4-5 donor blastoderms into the AOV of non-radioactive host blastoderms of the same stage.

were able to become incorporated into the tissues of the host and to differentiate together with the host cells. Thus, the transplantation procedures appeared to have no overt effect on the normal differentiation pathways of the opaca mesodermal cells. (See Fig. 3.7, page 157).

The same type of finding was made in the next control experiment in which the mesodermal cells from the AP were transplanted back into the AP.

Experiment 3.IV : (AP mesoderm transplanted into the AP) (Control)

Radioactively labelled mesodermal fragments were isolated from the AP of stage 8 donor blastoderms and were transplanted into the AP of non-radioactive host blastoderms of the same developmental stage (Fig. 3.8).

(Text continues on page 158)

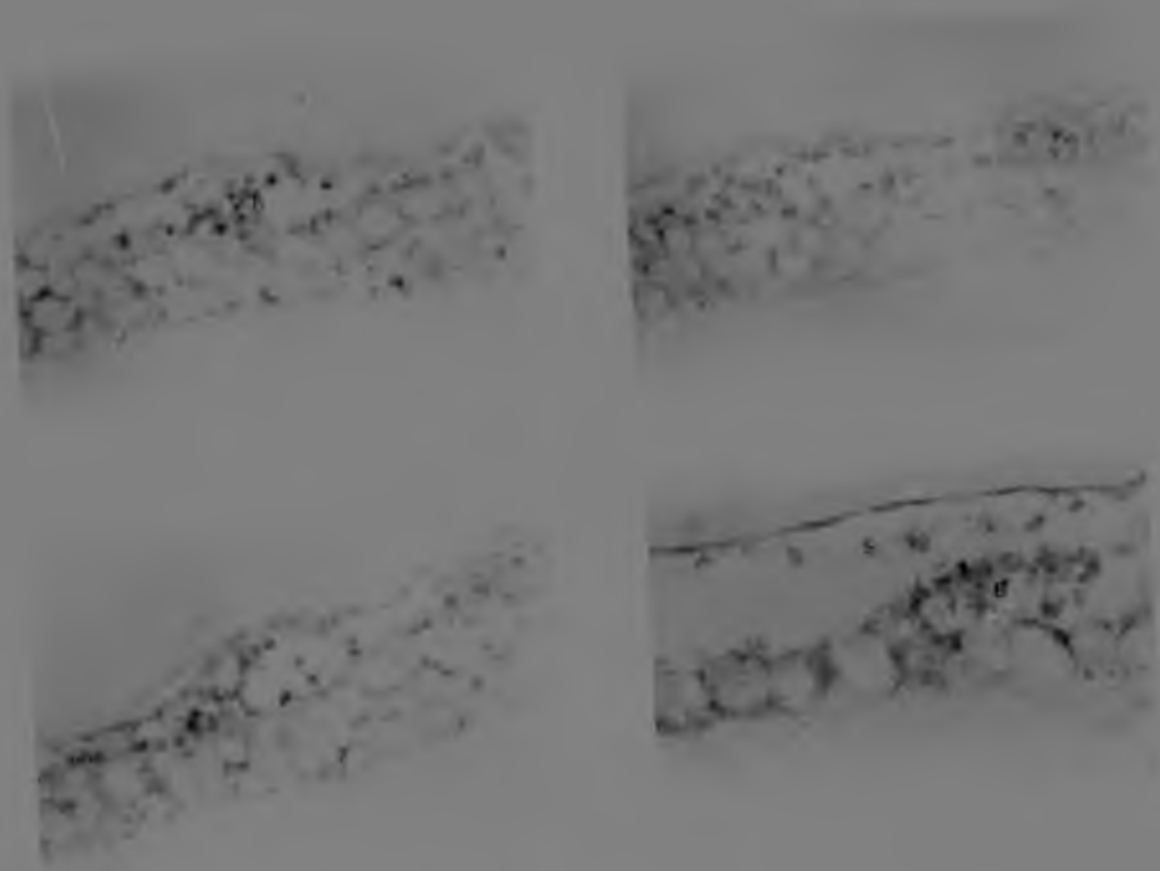


Figure 7.7

Autoradiographic histological sections from control Experiment 3.III in which radioactively labelled stage 4-5 opaca mesodermal fragments were transplanted into the AOV of non-radioactive stage 4-5 host blastoderms of the same stage. The labelled cells (i.e., transplanted mesodermal cells) are seen to have formed all the normal mesodermal cell types characteristic of the AOV, such as the intravascular blood cells (B), dorsal and ventral endothelial cells (E), and dorsal and ventral coelomic lining cells (S). Both labelled (i.e., donor) and unlabelled (i.e., host) cells are seen to have contributed to all the above structures.

B, intravascular blood cells; E, endothelial cells;
S, coelomic lining cells.

Top micrographs x 480
Bottom left x 480; Bottom right x 711.

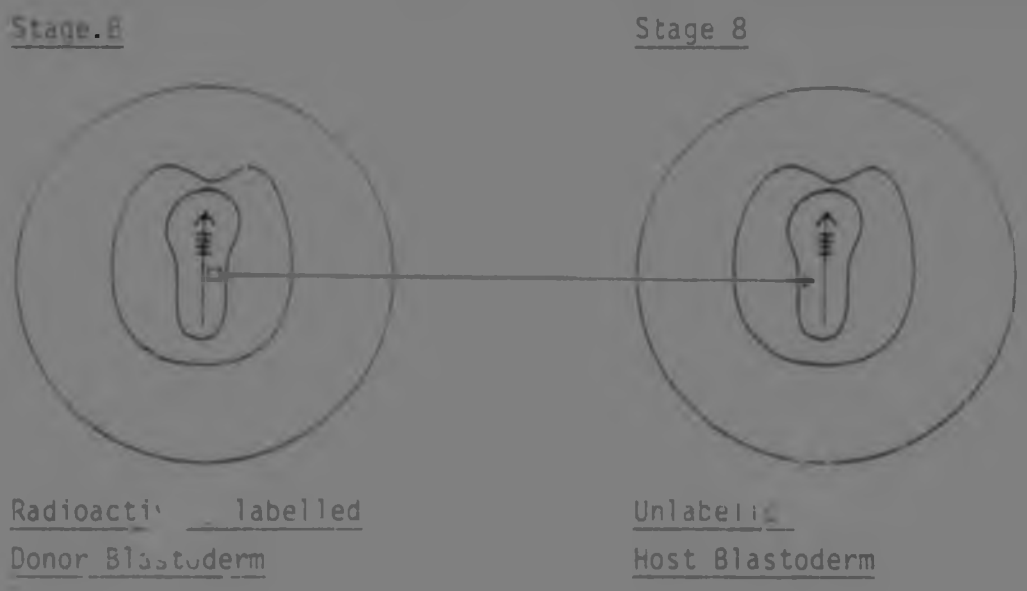


Figure 3.8

Diagram illustrating the transplantation of radioactively labelled mesodermal fragments from the AP of stage 8 donor blastoderms into the AP of non-radioactive host blastoderms of the same stage.

An examination of the autoradiographic histological serial sections revealed that the transplanted cells gave rise to all the normal mesodermal cell types characteristic of the AP. As shown in Fig. 3.9, the transplanted cells, together with the host cells, were seen to have given rise to the vessel endothelia, and the thick and thin coelomic linings. Furthermore, the donor mesodermal cells, like the host mesodermal cells, did not give rise to intravascular blood cells in the AP. This is in agreement with the observations from the histological study of the previous chapter that the blood vessels in the bulk of the AP are formed in the absence of the formation of intravascular blood cells.

In the present control experiment, as in the previous one, both labelled and unlabelled cells were found to have contributed to the formation of the various structures, indicating that the transplanted mesodermal cells were able to become incorporated into the tissues of the host and to differentiate together with the host cells.

Since in both control experiments the transplanted mesodermal cells became developmentally integrated with the surrounding differen-

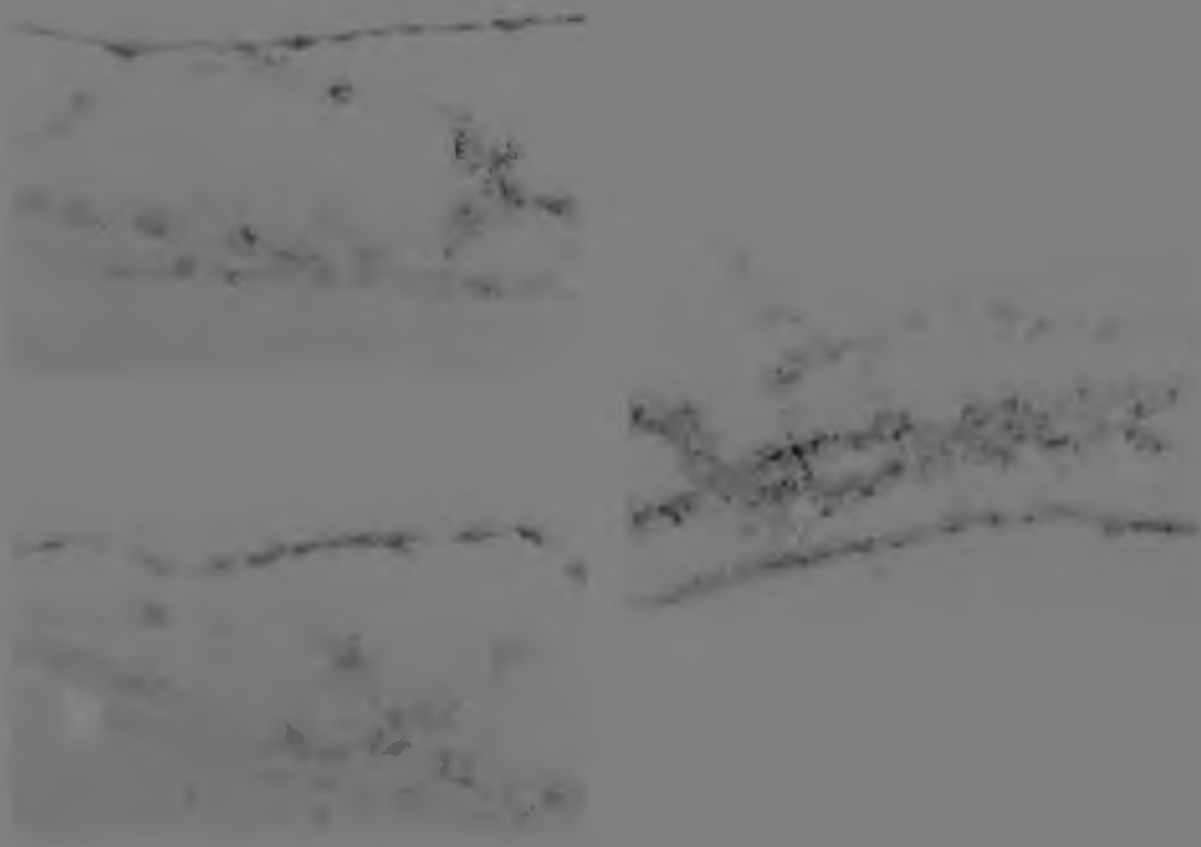


Figure 3.9

Autoradiographic histological sections from control Experiment 3.IV in which radioactively labelled stage 8 pellucida mesodermal fragments were transplanted into the AP of non-radioactive stage 8 host blastoderms. The labelled cells (i.e., transplanted mesodermal cells) are seen to have formed all the normal mesodermal cell types characteristic of the AP, such as the dorsal and ventral endothelial linings (E) and the dorsal and ventral coelomic linings (S). Both labelled (i.e., donor) and unlabelled (i.e., host) cells are seen to have contributed to all the above structures.

E, endothelial cells; S, coelomic lining cells.

x 711

tiating host cells, it is concluded that the transplantation procedures themselves had no overt effect on the pathways of differentiation of the mesodermal cells. It is therefore further concluded that the changes in the differentiation pathways observed in some of the pellucida and opaca mesodermal cells when they were heterotopically transplanted from one region of the blastoderm into another (i.e., from the AP into the AOV and vice versa) (Experiments 3.I and 3.II) were likewise not a consequence of the transplantation procedures, but rather, were probably due to the

environmental influences inherent in the different regions of the blastoderm.

3.4 Discussion

3.4.1 The pathways of differentiation of the heterotopically transplanted mesodermal cells

The objectives of the present study were to investigate whether the pathways of differentiation of the pellucida and opaca mesodermal cells would be altered if they were transplanted from one part of the blastoderm into another (i.e., from the AP into the AOV, and vice versa). The question being examined was whether the pellucida and opaca mesodermal cells are different from each other, or whether their pathways of differentiation are dependent upon the differential environmental influences inherent in the AP and AOV.

No clear-cut "yes" or "no" answer emerged from this study. Rather, it was found that whereas some of the heterotopically transplanted mesodermal cells differentiated in accordance with their original fates, others differentiated in conformity with their new environments.

The situations in which the mesodermal cells differentiated in accordance with their original fates are:

1. Some of the stage 8 pellucida mesodermal cells which had been transplanted into the AOV of stage 5 hosts were found to have given rise to dorsal and ventral endothelial cells and dorsal and ventral coelomic lining cells (Experiment 3.I). During normal development the pellucida mesodermal cells give rise to endothelial linings and coelomic linings in the AP. The present observations thus indicate that these cells are able to give rise to the same types of linings when they are heterotopically transplanted into the AOV.

2. Some of the stage 8 pellucida mesodermal cells which had been transplanted into the AOV of stage 5 hosts were found to have given rise to the walls of thick-walled vesicles (Experiment 3.I). These thick-walled vesicles resemble the thick-walled coelomic linings which are normally formed from the lateral plate mesoderm in the medial parts of the AP,

and are not normally formed in the AOV.

3. Some of the stage 4-5 opaca mesodermal cells which had been transplanted into the AP of stage 8 hosts were found to have given rise to large numbers of intravascular blood cells (Experiment 3.II). Large numbers of blood cells are not normally formed in the AP itself. The fact that the transplanted opaca cells gave rise to large numbers of blood cells in the AP suggests that many of the transplanted stage 4-5 opaca mesodermal cells were already determined in the direction of blood cell formation at the time of transplantation. This finding is in agreement with the fate maps of Settle (1954).

4. Some of the stage 4-5 opaca mesodermal cells which had been transplanted into the AP of stage 8 hosts were found to have moved out of the AP into the AOV of the hosts (Experiment 3.II). This situation is in contrast to the observations of the control experiment in which the stage 8 pellucida mesodermal cells which had been transplanted back into the AP of stage 8 hosts were seen to have remained in the AP of the hosts (Experiment 3.IV). This finding highlights the differences in the behavioural properties of the stage 4-5 opaca- and stage 8 pellucida mesodermal cells. In normal development the mesodermal cells of the stage 4-5 AOV are still actively migrating through the AOV, whereas the mesodermal cells of the stage 8 AP tend to remain in the AP as they embark upon their overt differentiation (see Chapter Two).

The situations in which the mesodermal cells differentiated in conformity with their new environments are:

1. Some of the stage 8 pellucida mesodermal cells which had been transplanted into the AOV of stage 5 hosts were found to have given rise to intravascular blood cells (Experiment 3.I). The pellucida mesodermal cells do not normally give rise to blood cells in the AP itself. (It could be argued, however, that the transplanted mesodermal fragments in the present experiment may have been taken from the 'intermediate zone' at the pellucida-opaca border where the mesodermal cells normally tend to give rise to small numbers of intravascular blood cells. However, in the present experiments care was taken to ensure that the mesodermal fragments were excised from the regions of the AP where blood cells do not normally form. Furthermore, in the control experiment in which

stage 8 pellucida mesodermal cells were isolated from the same region of the AP, but were transplanted back into the AP of stage 8 hosts, the transplanted cells did not give rise to intravascular blood cells in the AP of the hosts (Experiment 3.IV). Additional confirmational evidence that the pellucida mesodermal fragments were isolated from a normally non-erythropoietic region of the blastoderm is presented in the following chapter (Section 4.3.1) in which it is found that stage 8-9 pellucida fragments isolated from the same region of the blastoderm and cultured *in vitro* do not differentiate along erythropoietic lines).

2. A small number of the transplanted stage 4-5 opaca mesodermal cells which had been transplanted into the AP of stage 8 hosts appeared to have formed endodermal cells (Experiment 3.II). This observation is in line with the findings of Rosenquist (1966) that some of the mesenchymal cells in the AP enter into the underlying hypoblastic layer and give rise to the embryonic endoderm in the AP. The transplanted opaca mesodermal cells would not have formed endodermal cells if they had remained in the AOV.

In view of the finding that some of the transplanted mesodermal cells differentiated in accordance with their original fates whereas others differentiated in conformity with their new environments, it would appear that at the time of transplantation the pathways of differentiation of some of the stage 8 pellucida- and stage 4-5 opaca mesodermal cells had already been determined, whereas others were still developmentally plastic. This would suggest that the microenvironmental factors emanating from the particular regions of the blastoderm (i.e., the AP or AOV) may indeed be important in determining the pathways of differentiation of the mesodermal cells, provided the mesodermal cells are isolated at a time before their developmental fates have already become fixed.

This conclusion is in line with the findings from the classical transplantation experiments of Spemann (1919, 1921 - see Balinsky, 1931 page 258 *et sequ*). This author carried out heterotopical transplantations between the presumptive ectodermal regions in the early newt embryo and showed that up to a certain stage of development the pathway of differentiation of the ectodermal tissue was dependent upon its location in the embryo. At later stages, when the developmental fate of the ectoderm had become fixed, the transplanted tissue differentiated in accordance with its prospective significance regardless of its location in the embryo.

Furthermore, studies on the developmental fate of the mesodermal tissue in the amphibian embryo by Yamada (1937, 1940) and Muchmore (1951) have shown that all parts of the mesodermal mantle of the early urodele neurula are still capable of a wide range of differentiation. For example, presumptive blood-island material transplanted to the presumptive somite or pronephros regions differentiated along with the host cells into muscle or pronephric tubules, respectively. Likewise, presumptive pronephros transplanted into the somite or blood-island regions formed muscle or blood cells, according to its new location (Yamada, 1937).

Returning to the question of whether the pellucida and opaca mesodermal cells are different from each other or whether their pathways of differentiation are dependent upon environmental influences, it is suggested that the mesodermal cells of the early chick blastoderm are probably all originally pluripotent, but that the environment into which they will subsequently come to lie (i.e., the AP or AOV) will direct the pathways of differentiation upon which they will subsequently embark. Further aspects of the role of environmental factors on the differentiation pathways of the mesodermal cells are dealt with in the following chapters (Chapters Four to S x).

3.4.2 Discussion of the control experimen s

The two control experiments which were carried out verified that the transplantation procedures themselves were not the factor affecting the types of differentiation of the mesodermal cells. In these experiments (Experiments 3.III and 3.IV) the mesodermal fragments were isolated from the AP or AOV of donor blastoderms and were transplanted into the same regions of host blastoderms of the same developmental stages. It was found that the transplanted mesodermal cells differentiated together with the surrounding mesodermal cells in accordance with the normal differentiation pathways of the particular region of the blastoderm. Thus, stage 4-5 opaca mesoderm transplanted into the AOV of stage 4-5 hosts contributed to the formation of all the normal cell types characteristic of the AOV, viz., intravascular blood cells, endothelial cells, and coelomic lining cells (Experiment 3.III). Likewise, stage 8 pellucida mesoderm transplanted into the AP of stage 8 hosts contributed to the formation of all the normal cell types characteristic of the AP, viz., vessel endothelia, and thick and tnin coelomic linings (Experiment 3..V). The transplanted cells, like the host mesodermal cells, did

not give rise to structures which were not characteristic of the particular region of the blastoderm. For example, the pellucida mesodermal cells which were transplanted into the AP of the hosts did not give rise to blood cells in the AP of the hosts. This is in agreement with the fact that during normal development the mesodermal cells in the bulk of the AP do not give rise to blood cell (see Chapter Two).

Since in both control experiments the transplanted mesodermal cells became developmentally integrated with the surrounding mesodermal cells, it appears that the transplantation procedures themselves had no overt effect on the pathways of differentiation of the mesodermal cells. It is therefore concluded that the changes in the differentiation pathways observed when the pellucida or opaca mesodermal cells were heterotopically transplanted into the AOV or AP of the hosts (Experiments 3.I and 3.II) were probably not due to the technical aspects of the transplantation procedures, but rather, were probably a consequence of the environmental influences inherent in the different regions of the blastoderm.

3.4.3 Discussion of the experimental techniques

As described in Section 3.2 and 3.3, the experimental technique employed in the present investigation involved the isolation of radioactively labelled mesodermal fragments from the AOV or AP of radioactively labelled donor blastoderms of the required age, and their transplantation into the AOV or AP of non-radioactive host blastoderms of the required age. The host blastoderms were then cultured overnight on solid organ culture medium, and the fate of the labelled cells (i.e., the transplanted cells) was followed by autoradiography of histological serial sections.

An alternative technique to that of transplanting radioactively labelled cells and following their fate autoradiographically, involves the use of cell-markers, based on the histological differences between the interphase nuclei of the chick and the Japanese quail (Le Douarin, 1973a,b). The cells of the Japanese quail contain a large amount of heterochromatic DNA associated with the nucleolus. This can be visualized by Feulgen-Rossenbeck staining as one or more large chromocentres. In the cells of the chick, on the other hand, the chromatin is evenly

distributed in the nucleoplasm during interphase, and forms a fine network with only small dispersed chromocentres. Owing to these differences between the nuclei of quail and chick cells, quail cells have been used as natural markers when combined [illegible] with chick cells (Le Douarin, 1973a,b).

In the present study this chick-quail marker technique was to have been employed in the tissue transplantation experiments, transplanting quail donor cells into chick host blastoderms (and vice versa). However, when this method was carried out it was found that the quail cells could only be definitely identified in the chimaeric tissue when there were numbers of quail cells in a given location. If, however, the quail cells were sparcely distributed amongst the chick cells, then difficulty was experienced in finding the quail cells readily when scanning through numerous serial sections. Furthermore, it was often difficult for this author to unequivocally identify the quail cells when they occurred singly in an area of mainly host (i.e., chick) cells. On the other hand, when ^{3}H-thymidine labelled donor cells were employed, the transplanted cells were readily identifiable from the host cells due to the obvious presence of black silver-grains over their nuclei in autoradiographical histological sections (Figs. 3.2, 3.3, 3.5, 3.7, 3.9). For these reasons, radioactively labelled donor cells, and autoradiographical methods for their visualisation, were employed in the present study.

CHAPTER FOUR

AN IN VITRO INVESTIGATION OF THE EFFECTS OF THE COMPONENT CELL TYPES OF THE AREA PELLUCIDA AND AREA OPACA VASCULOSA ON THE ERYTHROPOIETIC DIFFERENTIATION OF THE MESODERMAL CELLS, BY MEANS OF THE COVERSLIP CULTURE OF BLASTODERM FRAGMENTS IN SMALL DROPS OF MEDIUM

4.1 Introduction

A major issue being dealt with in the present thesis is the question of the pluripotency of the mesodermal cells and the extent to which environmental factors affect their pathways of differentiation. This aspect has been highlighted by the findings from the tissue transplantation experiments of the previous chapter that some of the heterotopically transplanted mesodermal cells differentiate along developmental pathways which are different from their original fates, but are in conformity with their new environments. For example, it is found that some of the stage 8 pellucida mesodermal cells, which *in situ* do not normally form blood cells, give rise to blood cells when they are transplanted into the AOV of stage 5 host blastoderms (see Chapter Three). In the present chapter a different type of experimental approach is employed in an attempt to evaluate the contributions of the different germinal layers in the different regions of the blastoderm (i.e., the ectoderm, mesoderm and endoderm of the AP and AOV, respectively) to the pathways of differentiation of the mesodermal cells. This study investigates the effects of these component cell types on the erythropoietic differentiation of the mesodermal cells. The technique employed is a coverslip culture method, and the extent of the erythropoietic differentiation of the mesodermal cells is monitored by the o-dianisidine staining method for the detection of haemoglobin (O'Brien, 1961).

In these experiments fragments of tissue composed of various combinations of the germinal layers from the AP or AOV of blastoderms of known ages (e.g., 0,5mm x 0,5mm fragments of ectoderm-plus-mesoderm; mesoderm alone; etc.) were cultured either alone or in combination with fragments from the AP or AOV of other blastoderms. The explants were cultured for 72 hours in a small drop of culture medium on a cover-

slip, below a cavity slide (see Materials and Methods below). Thereafter, the explants were stained with the o-dianisidine stain (O'Brien, 1961) which stains haemoglobin brown[1]. The results of these experiments have been grouped into five classes, representing increasing degrees of haemoglobin staining intensity (see key, Fig. 4.1 page 171). This enables a comparison to be made between the erythropoietic differentiation of the mesodermal cells cultured in different combinations of the other germinal layers.

The following series of test cultures were carried out to examine the interactions between the different germinal layers of the AP and AOV on mesodermal erythropoiesis:

1. Routine control cultures which were carried out to ensure that the culture medium and the o-dianisidine stain were always optimal:
Expt 4.I : ecto + meso + endo (AOV, st 8-9) = control;

2. Cultures to investigate the effects of the stage 8-9 pellucida ectoderm and/or endoderm, and the stage 4 opaca endoderm, on the erythropoietic differentiation of the stage 8-9 pellucida mesoderm (Section 4.3.1):
Expt 4.II : meso (AP, st 8-9);
Expt 4.III : ecto + meso (AP, st 8-9);
Expt 4.IV : ecto + meso + endo (AP, st 8-9);
Expt 4.V : meso (AP, st 8-9) + endo (AOV, st 4);
Expt 4.VI : ecto + meso (AP, st 8-9) + endo (AOV, st 4);
Expt 4.VII : endo (AOV, st 4) = control;

3. Cultures to investigate the effects of the stage 4 opaca ectoderm and/or endoderm on the erythropoietic differentiation of the stage 4 opaca mesoderm (Section 4.3.2):
Expt 4.VIII : ecto + meso (AOV, st 4) + endo (AOV, st 4);
Expt 4.IX : ecto + meso (AOV, st 4);
Expt 4.X : meso (AOV, st 4);
Expt 4.XI : meso (AOV, st 4) + endo (AOV, st 4);
Expt 4.VII : endo (AOV, st 4) = control;

---

[1] The o-dianisidine method is a histochemical technique which utilizes the peroxidase activity of haemoglobin. Haemoglobin reacts with hydrogen peroxide as substrate and o-dianisidine as the H-donor or 'acceptor' to convert the latter into an orange-brown dye by peroxidatic oxidation (O'Brien, 1961).

4. Cultures to investigate the effects of the stage 4 pellucida ectoderm and/or endoderm, and the stage 4 opaca endoderm, on the erythropoietic differentiation of the stage 4 pellucida mesoderm (Section 4.3.3.):

Expt 4.XII : meso (AP, st 4);
Expt 4.XIII : ecto + meso (AP, st 4);
Expt 4.XIV : ecto + meso + endo (AP, st 4);
Expt 4.XV : meso (AP, st 4) + endo (AOV, st 4);
Expt 4.VII : endo (AOV, st 4) = control;

5. Cultures to investigate the effects of the stage 1-2 opaca ectoderm and/or endoderm on the erythropoietic differentiation of the stage 4- and stage 8-9 pellucida mesoderm (Section 4.3.4):

Expt 4.XVI : meso (AP, st 4) + endo (AOV, st 1-2);
Expt 4.XVII : meso (AP, st 8-9) + endo (AOV, st 1-2);
Expt 4.XVIII : meso (AP, st 4) + ecto + endo (AOV, st 1-2);
Expt 4.XIX : meso (AP, st 8-9) + ecto + endo (AOV, st 1-2);
Expt 4.XX : endo (AOV, st 1-2) = control;
Expt 4.XXI : ecto + endo (AOV, st 1-2) = control.

Comparing the extent of erythropoiesis which occurs in these different combinations of the germinal layers, relevant findings have emerged with respect to the interactions between the three germinal layers during mesodermal erythropoiesis. These observations have bearing on the graded pattern of erythropoiesis which occurs in the AOV and AP during normal embryogenesis (see Chapter Two).

4.2 Materials and Methods

4.2.1 The coverslip culture of blastoderm fragments in small drops of culture medium

All the procedures for the coverslip culture experiments were carried out under sterile conditions, as described in Section 3.2.1. Small fragments of the blastoderm, composed of given combinations of the germinal layers, were explanted into a small drop of culture medium on the surface of coverslips, below cavity slides, as detailed below.

4.2.1.1 Preparation of blastoderm fragments

Chick blastoderms of the required incubation stages (see experiments below) were dissected from the yolk in chick embryo saline and freed from their vitelline membranes. The blastoderms were then transported to a petri dish of saline on the stage of a binocular microscope for further operational procedures.

0,5mm x 0,5mm fragments of the blastoderms in various combinations of the germinal layers (e.g., ectoderm-plus-mesoderm; mesoderm alone; mesoderm-plus-endoderm; endoderm alone) were dissected from selected areas of the blastoderms as required (see experiments below). Sharp stainless-steel insect pins (D.F. Tayler and Co., England, size 0) mounted on pencil-length glass rods were used for peeling the endoderm from the underlying mesoderm and ectoderm. A fine hair-loop made with infants' hair (Rugh, 1962 page 8) was used for scraping the mesodermal layer off the ectoderm. Where necessary, the dissected tissue was cut into fragments by working the tips of the insect pins against each other in a shearing motion.

The blastoderm fragments were transported into the drop of liquid culture medium (Section 4.2.1.2) on a coverslip (Section 4.2.1.3) by means of a micro-syringe with a vernier control (Burroughs Wellcome and Company, England; and G.K.N. Shardlow Metrology Ltd., England). Where required, the different types of fragments were then sandwiched together (see experiments below), using the insect pins and hair loops to manipulate the tissues.

4.2.1.2 Preparation of the liquid culture medium

The liquid culture medium was the same as that used by Hagopian *et al* (1972). The culture medium consisted of seven parts of Ham Nutrient Mixture F12 with L-glutamine (GIBCO), one part of a particular batch of heat-inactivated (56°C, 30 mins.) foetal calf serum (GIBCO), 100 units per ml penicillin-G-sodium (Novo) and 100µg per ml streptomycin sulphate (Hoechst).

Prior to dispensing the solutions, the openings of the bottles were swabbed with 70% ethanol and flamed with a spirit burner.

4.2.1.3 Method of coverslip culture

A 0,03 ml drop of liquid culture medium (prepared as described in Section 4.2.1.2 above) was syringed into the centre of 20mm x 20mm glass coverslips (Chance) in individual petri dishes. The glass coverslips had previously been cleaned and siliconized as follows: The coverslips were immersed overnight in 2N HCl, washed in ten changes of tap water followed by three changes of double glass-distilled water, rinsed in 95 ethanol and allowed to air-dry in a dust-free drying oven. The coverslips were thereafter siliconized by a fifteen-seconds immersion in 2% dichlordimethylsilan (Merck) in carbon tetra-chloride (Analar) and baked for three hours in a 105°C oven.

The dissected blastoderm fragments (prepared as described in Section 4.2.1.1 above) were transported in saline into the drop of culture medium by means of the vernier-controlled micro-syringe. Owing to the slight dilution of the culture medium with the saline during the transportation procedure, the culture medium was renewed by sucking off half the volume of the drop with the vernier-controlled micro-syringe, and replacing it with fresh culture medium. This was repeated.

Cavity slides (cavity depth, 3mm at all points; diameter, 14mm; rim, 6mm wide for even support of the coverslip) were lowered onto the surface of the coverslips so that they surrounded the drop of culture medium. Before being placed in position, a thin film of petroleum jelly (Vaseline) was applied to the rim of the cavity slides to make an air-tight seal between the rim of the cavity slide and the coverslip. This prevented moisture-loss from the resultant culture chambers.

The coverslips-plus-cavity slides were maintained in petri dishes lined with moist cottonwool, and the tissue fragments were cultured as such in small drops of medium at 38°C for 72 hours.

4.2.1.4 Fixation, staining and processing of the coverslip cultures

After the 72 hour culturing period, the explants were fixed, stained and processed as follows: The coverslips were separated from the cavity slides and placed on the stage of a binocular microscope

(Text continues on page 172).



\-

Blue staining of counterstain only (i.e., no haemoglobin-containing cells).



-+

Traces of pale yellow staining (i.e., staining for haemoglobin uncertain).



\+

Pale brown staining in few to many cells (i.e., pale haemoglobin staining in a variable number of cells).



Very intense brown staining in a small number of cells (i.e., very intense haemoglobin staining in a small number of cells), or



++

Brown staining in a large number of cells (i.e., haemoglobin staining in a large number of cells).



+++

Very intense brown staining in a large number of cells (i.e., very intense haemoglobin staining in a large number of cells).

Figure 4.1

Key representing increasing intensities of haemoglobin staining, as judged by the colour produced by the o-dianisidine stain. The o-dianisidine stain stains haemoglobin brown. The counterstain (methyl green) stains the rest of the tissue blue.

x 80

in front of a lamina flow unit. A vernier-controlled micro-syringe was used to remove excess culture medium from the drop, care being taken not to remove any cells. A double volume of o-dianisidine fixative-stain was then added to the culture with the vernier-controlled micro-syringe. The o-dianisidine fixative-stain was freshly prepared from 4 parts o-dianisidine stock (100mg o-dianisidine (BDH) in 70ml absolute ethanol); 1 part 0,1M sodium acetate, pH 4,6; 1,5 parts double glass-distilled water; 0,2 parts hydrogen peroxide (30%) (BDH).

The stained coverslip preparations were air dried in front of the lamina flow unit. Thereafter, the preparations were washed in two changes of double distilled water, counterstained in 1% methyl green for 1 minute, rinsed briefly in double distilled water, dehydrated in a graded series of p-dioxane, cleared in xylol, and mounted with DPX on precleaned glass slides.

The extent of erythropoiesis of the explants was scored by the intensity of o-dianisidine staining (Fig. 4.1).

4.3 Experiments and Results

4.3.1 An investigation of the ability of the stage 8-9 pellucida mesodermal fragments to give rise to blood cells when cultured alone or in combination with the other germinal layers from the AP and AOV - (Experiments 4.I to 4.VII)

As outlined above, an important finding from the tissue transplantation experiments of the previous chapter (Chapter Three) is that some of the stage 8 pellucida mesodermal cells, which *in situ* do not normally form blood cells, gave rise to blood cells when they were transplanted into the AOV of stage 5 host blastoderms. This would suggest that some of the stage 8 pellucida mesodermal cells are still pluripotent, in that their pathways of differentiation can still be changed if they are subjected to the environmental factors of the AOV. The following coverslip culture experiments were performed in an attempt to verify these findings and to analyse the effects of the component cell types of the AP and AOV on the erythropoietic differentiation of the stage 8 pellucida mesodermal cells. In this study an investigation has

been made of the ability of the stage 8-9 pellucida mesodermal cells to form blood cells when cultured alone or in the presence of the other germinal layers from the AP or AOV.

The following test cultures were performed (as described in detail below):

Expt 4.I : ecto + meso + endo (AOV, st 8-9) = control;
Expt 4.II : meso (AP, st 8-9);
Expt 4.III : ecto + meso (AP, st 8-9);
Expt 4.IV : ecto + meso + endo (AP, st 8-9);
Expt 4.V : meso (AP, st 8-9) + endo (AOV, st 4);
Expt 4.VI : ecto + meso (AP, st 8-9) + endo (AOV, st 4);
Expt 4.VI' : endo (AOV, st 4) = control.

Each of the culture experiments was repeated a large number of times, the minimum number of repeats being ten. The results of these experiments have been tabulated in Table 4.1. As outlined above, the results of these experiments have been grouped into 5 classes, representing increasing degrees of haemoglobin staining intensity (see key, Fig. 4.1). The t-test was employed to make statistically valid comparisons between the extent of the erythropoietic differentiation of the explants in which different combinations of the germinal layers were employed (e.g., Experiment 4.II compared with Experiment 4.IV, etc.). Values of p equal to or lower than 0,05 were taken as indicating significant differences between the results of the experiment pairs (see Roscoe, 1975; Parker, 1979).

As part of every culture experiment, control explants isolated from a normally erythropoietic part of the blastoderm were routinely cultured and stained for haemoglobin (Experiment 4.I). These controls were carried out to ensure that the culture medium and the o-dianisidine stain were always optimal.

An outline of the experiments (including controls) performed, and the results obtained, is presented below.

4.3.1.1 Culture experiments

Experiment 4.I (ecto + meso + endo (AOV, st 8-9))
(Control)

As indicated above, control explants excised from a potentially

erythropoietic part of the blastoderm were routinely cultured during all experimental runs to ensure that the culture medium and the o-dianisidine stain were always optimal. 0,5mm x 0,5mm fragments composed of all three germinal layers (i.e., ectoderm, mesoderm and endoderm) were isolated from the posterio-lateral AOV of stage 8-9 blastoderms (Fig. 4.2). This is the region of the blastoderm in which blood cells would later form (see Chapter Two). The fragments were cultured for 72 hours in small drops of culture medium, as described above, and thereafter the haemoglobin was stained with the o-dianisidine stain. As tabulated in Table 4.1, 100% of these explants (167 cases out of 167) were found to be in category +++, exhibiting very intense brown staining in a large number of cells. This indicated that the culture conditions were indeed healthy, and that the o-dianisidine stain was optimal.

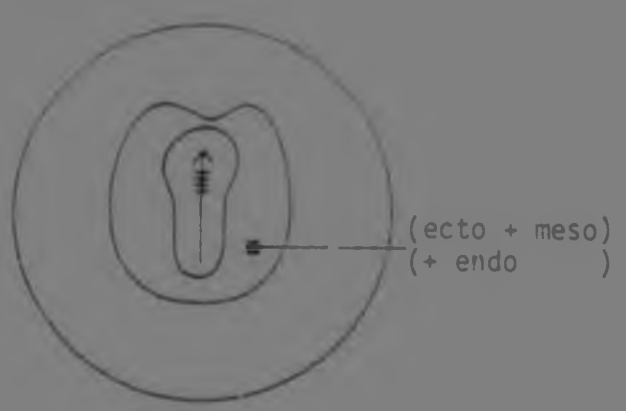


Figure 4.2

Diagram illustrating the area from wnere fragments composed of the three germinal layers (i.e., ectoderm, mesoderm and endoderm) were isolated from the AOV of stage 8-9 blastoderms.

Experiment 4.II (meso (AP, st 8-9))

In this experiment the ability of the isolated stage 8-9 pellucida mesodermal fragments to differentiate erythropoietically was investigated. 0,5mm x 0,5mm mesodermal fragments were isolated from the AP of stage 8-9 blastoderms (Fig. 4.3). These fragments were isolated from the same region from where the pellucida mesodermal fragments were isolated in the transplantation experiments of the previous chapter

(Experiments 3.I and 3.IV, Chapter Three).

Stage 8-9

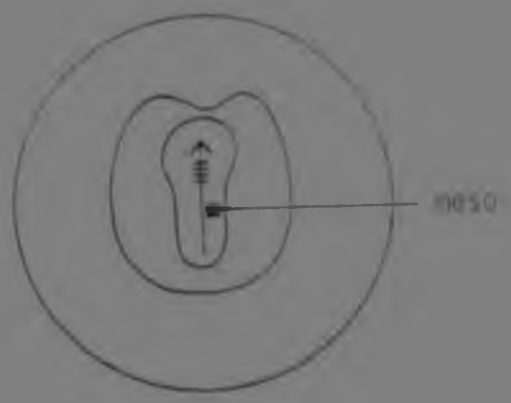


Figure 4.3

Diagram illustrating the area from where mesodermal fragments were isolated from the AP of stage 8-9 blastoderms.

After 72 hours of culture in small drops of culture medium, the following results were obtained, as tabulated in Table 4.1.

- \- In 87% of the explants (67 cases out of 77) the staining for haemoglobin was negative;
- -+ In 7,8% of the explants (6 cases out of 77) there were traces of pale yellow staining (i.e., uncertain haemoglobin staining);
- \+ In 5,1% of the explants (4 cases out of 77) there was pale brown (i.e., pale haemoglobin) staining in a small number of cells.

An interesting point to note is that not all of the 'negative' explants stained the blue colour of the counterstain only. Instead, 29 of the 67 'negative' cultures contained some cells which stained a pale pink colour. Furthermore, many of these pink staining cells tended to be free-floating and spindle- or boat-shaped (Fig. 4.4, left hand micrograph). This spindle- or boat-shape morphology is similar to the shape of many of the free floating erythrocytes typical of +++ cultures, which are intensely stained for haemoglobin (Fig. 4.4, right hand micrograph).



Figure 4.4

The left hand micrograph shows a few cells from an explant of stage 8-9 pellucida mesoderm after 72 hours of culture and subsequent o-dianisidine staining. This is an example of the observations made in 29 of the 67 'negative' explants in which some of the cells stained a pale pink colour. Many of these pink staining cells tended to be free floating and spindle- or boat-shaped (arrows). This spindle- or boat-shape morphology is characteristic of the shape of many of the free floating erythrocytes in typical +++ cultures, which are intensely stained for haemoglobin (right hand micrograph). The right hand micrograph shows some free floating erythrocytes of a typical +++ culture. Note that many of the cells are spindle- or boat-shaped (arrows).

x 711

Experiment 4.III (ecto + meso (AP, st 8-9))

For comparison, the next experiment was carried out in which the erythropoietic potential of stage 8-9 pellucida mesoderm cultured in combination with its own pellucida ectoderm was investigated. Stage 8-9 pellucida fragments composed of ectoderm-plus-mesoderm were isolated from the same region of the AP (Fig. 4.5) and cultured for 72 hours in small drops of culture medium.

The following results were obtained, as tabulated in Table 4.1.

- 90% of the explants (18 cases out of 20) stained the blue colour of the counterstain only (i.e., the staining for haemoglobin was negative);
-+ 10% of the explants (2 cases out of 20) exhibited traces of pale yellow staining (i.e., uncertain haemoglobin staining).

Stage 8-9

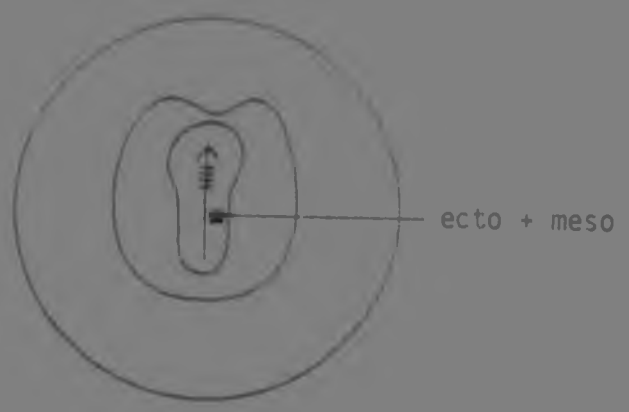


Figure 4.5

Diagram illustrating the area from where ectoderm-plus-mesodermal fragments were isolated from the AP of stage 8-9 blastoderms.

None of the present explants stained the pale pink colour or exhibited the free floating boat-shaped cells as occurred in the previous experiment in which stage 8-9 pellucida mesodermal fragments were isolated and cultured alone (Experiment 4.II).

In the present experiment it was found that part of the explants tended to form a balloon-like epithelial vesicle which, by focusing the microscope at different planes, was seen to enclose a clump of cells (Fig. 4.6). In some cases, a second clump of cells was also found on the outside of the vesicle (Fig. 4.6). In terms of the behaviour of these cell types, it is probable that the epithelial vesicles were formed from the ectodermal portion of the ectoderm-plus-mesodermal explants, whereas the clumps of cells were probably mesodermal in origin. (An unequivocal identification of the origin of the cells forming the vesicles and clumps could be obtained by combining ^{3}H-labelled ectoderm with unlabelled mesoderm (or vice versa) and tracing the fates of the different cell types in autoradiographical histological sections).

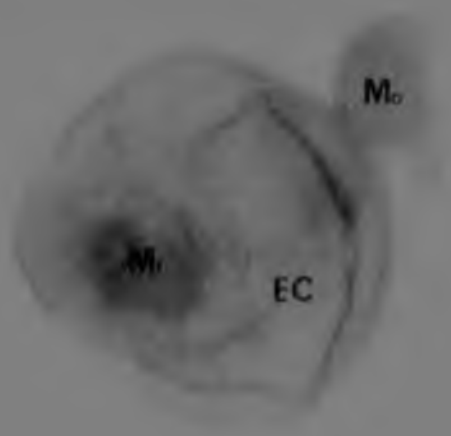


Figure 4.6

Photomicrograph of a stage 8-9 pellucida ectoderm-plus-mesodermal explant after 72 hours of culture. Part of the explant appears to have behaved like the ectodermal portion of the explant, having formed a balloon-like epithelial vesicle (EC). By focusing the microscope at different planes, it is seen that this balloon-like vesicle has enclosed a clump of cells (Mi), whereas a second clump of cells (Mo) is found on the outside of the vesicle. In terms of the behaviour of these cell types, it is possible that EC is the ectodermal portion of the ectoderm-plus-mesodermal explant, whereas Mi and Mo are probably the mesodermal portions of the explant.

x 177

Experiment 4.IV (ecto + meso + endo (AP, st 8-9))

In this experiment the erythropoietic potential of explants composed of all three germinal layers of the AP was investigated. 0,5mm x 0,5mm fragments composed of the three germinal layers (i.e., ectoderm, mesoderm and endoderm) were isolated from the same region of the AP (Fig. 4.7) and cultured in small drops of culture medium as above.

After 72 hours of culture, the following results were obtained, as tabulated in Table 4.1.

- 88,9% of the explants (64 cases out of 72) stained the blue colour of the counterstain only (i.e., the staining for haemoglobin was negative);

-+ 5,6% of the explants (4 cases out of 72) exhibited traces of pale yellow staining (i.e., uncertain haemoglobin staining);

+ 5,6% of the explants (4 cases out of 72) exhibited pale brown (i.e., pale haemoglobin) staining in a small number of cells.

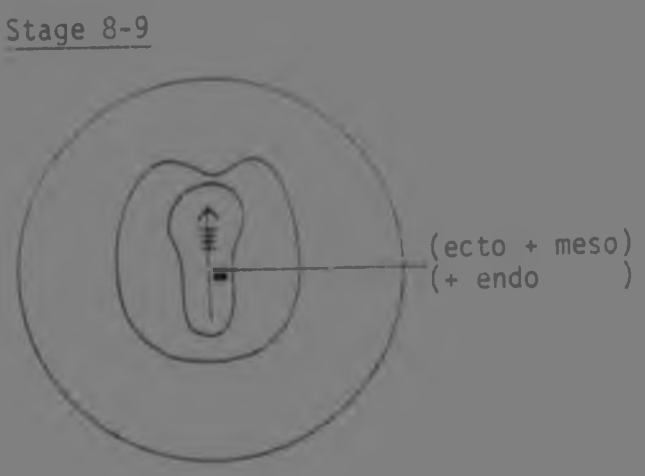


Figure 4.7

Diagram illustrating the area from where fragments composed of the three germinal layers (i.e., ectoderm, mesoderm and endoderm) were isolated from the AP of stage 8-9 blastoderms.

Again, none of the explants were found to contain the pink, free floating boat-shaped cells as occurred when stage 8-9 pellucida mesodermal fragments were isolated and cultured alone (Experiment 4.II). Rather, the ectoderm-plus-mesoderm-plus-endodermal explants were seen to have formed epithelial vesicles or linings which completely enclosed all the other cell types of the explants. The extent of haemoglobin staining in the present explants (i.e., stage 8-9 pellucida explants composed of all three germinal layers) is not significantly different from the results of the previous experiment in which stage 8-9 pellucida ectoderm-plus-mesodermal fragments (in the absence of the pellucida endoderm) were cultured (Experiment 4.III) (p = greater than 0,5).

Summarizing the results of the above three experiments, it is seen that stage 8-9 pellucida mesodermal fragments cultured alone (Experiment 4.II) or in combination with the pellucida ectoderm (Experiment 4.III), or the pellucida ectoderm-plus-endoderm (Experiment 4.IV) exhibit very low levels of haemoglobin synthesis, or no haemoglobin synthesis. None of the explants exhibited dark haemoglobin staining (categories ++ or +++). These findings are in agreement with the fact that the stage 8-9 pellucida mesodermal cells do not normally differentiate along erythropoietic lines (see Chapters Two and Three).

In the next pair of experiments (Experiments 4.V and 4.VI) the erythropoietic potential of stage 8-9 pellucida fragments in combination with stage 4 opaca endoderm was investigated. These experiments were carried out in view of the finding from the transplantation experiments of the previous chapter that the stage 8 pellucida mesodermal fragments gave rise to intravascular blood cells when they were transplanted into the AOV of stage 4-5 host blastoderms (Experiment 3.I, Chapter Three). The following two culture experiments were therefore performed to determine whether the erythropoietic differentiation of stage 8-9 pellucida mesoderm would be enhanced if it were cultured in combination with the stage 4 opaca endoderm. In this regard, Wilt (1965a) and Miura and Wilt (1969) had found that the erythropoietic differentiation of stage 4 opaca ectoderm-plus-mesodermal fragments was enhanced when they were cultured in the presence of stage 4 opaca endoderm.

Experiment 4.V (meso (AP, st 8-9) + endo (AOV, st 4))

Stage 8-9 pellucida mesodermal fragments were isolated from the same region of the AP as before, and combined with stage 4 opaca endodermal fragments isolated from the posterio-lateral region of the AOV (Fig. 4.8).



Figure 4.8

Diagram illustrating the areas from where stage 8-9 pellucida mesodermal fragments and stage 4 opaca endodermal fragments were isolated for combined culture.

After 72 hours of culture in small drops of medium, the follcwing results were obtained, as tabulated in Table 4.1.

- 29,6% of the explants (8 cases out of 27) stained the blue colour of the counterstain only (i.e., the staining for haemoglobin was negative);
-+ 11,1% of the explants (3 cases out of 27) exhibited traces of pale yellow staining (i.e., uncertain haemoglobin staining);
+ 14,8% of the explants (4 cases out of 27) exhibited pale brown (i.e., pale haemoglobin) staining in a small number of cells;
++ 44,4% of the explants (12 cases out of 27) exhibited intense brown staining (i.e., very dark haemoglobin staining) in a small number of cells.

Comparing the results of this experiment with those of Experiment 4.II, in which stage 8-9 pellucida mesodermal fragments were cultured alone (see Table 4.1), it is seen that the erythropoietic potential of the stage 8-9 pellucida mesoderm is indeed enhanced when it is cultured in the presence of the stage 4 opaca endoderm (Experiment 4.V) compared to when it is cultured alone (Experiment 4.II). The difference between the results of Experiments 4.II and 4.V is highly significant (p = very much less than 0,001).

Experiment 4.VI (ecto + meso (AP, st 8-9) plus)
(endo (AOV, st 4))

This experiment was carried out to investigate the effects of the stage 4 opaca endoderm on the erythropoietic differentiation of stage 8-9 pellucida ectoderm-plus-mesodermal fragments. Stage 8-9 pellucida ectoderm-plus-mesodermal fragments were isolated from the same region of the blastoderm as above, and combined with stage 4 opaca endodermal fragments isolated from the posterio-lateral region of the AOV, as above (Fig. 4.9).

After 72 hours of culture in small drops of medium, the following results were obtained, as tabulated in Table 4.1.

- 64,7% of the explants (11 cases out of 17) stained the blue colour of the counterstain only (i.e., the staining for haemoglobin was negative);
-+ No explants fell into this category,
+ 5,9% of the explants (1 case out of 17) exhibited pale brown (i.e., pale haemoglobin) staining in a small number of cells;

++ 29,4% of the explants (5 cases out of 17) exhibited intense brown staining (i.e., positive haemoglobin staining) in a small number of cells.

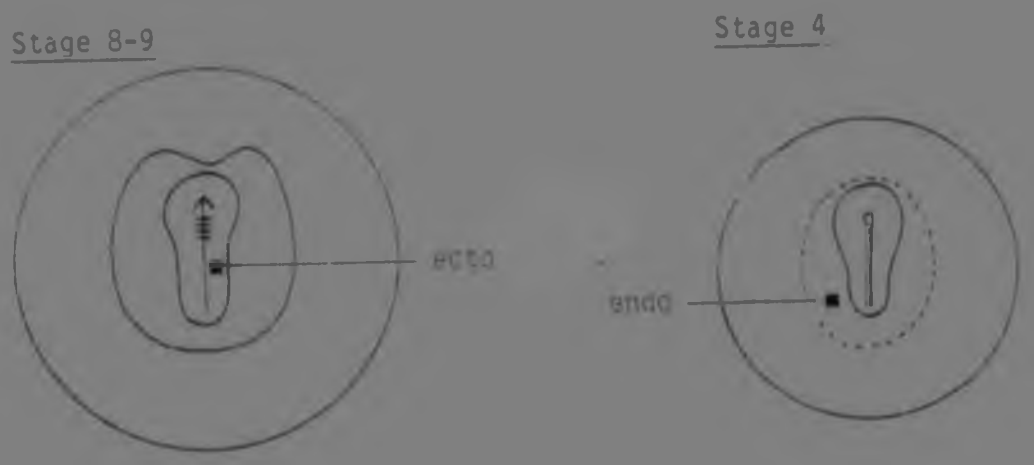


Figure 4.9

Diagram illustrating the areas from where stage 8-9 pellucida ectoderm-plus-mesodermal fragments and stage 4 opaca endodermal fragments were isolated for combined culture.

It was found that the opaca endodermal fragments and the pellucida ectoderm-plus-mesodermal fragments were easily recognizable from each other at the end of the culturing period. The endodermal tissue, identifiable owing to the refractile nature of its yolky cells, tended to form a cellular layer, like a carpet, over the surface of the coverslips. The ectoderm-plus-mesodermal fragments were non-refractile, and tended to form balloon-like vesicles which enclosed a central mass of cells in their interior (see Fig. 4.6). In some cases the ectoderm-plus-mesodermal portions were found to have become completely separated from the endodermal portions during the culturing period. These cultures were therefore discarded. Only those explants in which the ectoderm-plus-mesodermal fragments continued to maintain physical contact with the endodermal tissue throughout the culturing period were scored.

The development of the stage 8-9 pellucida ectoderm-plus-mesodermal fragments into vesicles enclosing cellular masses is identical to the situation which occurred in previous explants in which stage 8-9 pellucid ectoderm-plus-mesodermal fragments were cultured (Experiment 4.III) (Fig. 4.6). As suggested previously (based on the type of behaviour

of the cells) the vesicles are probably formed from the ectodermal portion of the explants and the cell masses are probably the mesodermal portion. However, an unequivocal identification of the origins of these cell types would have to be obtained experimentally, by combining ^{3}H-labelled ectoderm with unlabelled mesoderm (and vice versa) and tracing the fates of the cells in autoradiographical histological sections.

In the present experiments it was found that in the majority of cases (64,7% of the explants) the epithelial vesicle appeared to enclose completely the inner mass of cells, physically separating these inner cells from the underlying endoderm (Fig. 4.10, left hand diagram). In all these cases the staining for haemoglobin was negative. It is of interest to note that in approximately half of these cultures the ectoderm-plus-mesodermal portions, although still in contact with the endoderm at the end of the culturing period, tended to float upwards and become detached from the underlying endoderm during the subsequent processing procedures. In all cases the detached ectoderm-plus-mesodermal portions and the underlying endodermal portions exhibited negative staining for haemoglobin.

In the remaining 35,3% of the explants the epithelial vesicle did not completely enclose an inner mass of cells. In these explants clumps of cells were also found on the outside of the vesicles and in contact with the underlying endoderm (Fig. 4.10, right hand diagram). Haemoglobin synthesis tended to occur in a small number of the cells in the tissue which was in contact with the endoderm. It is of interest to note that in 2 of these 5 'haemoglobin positive' explants the ectoderm-plus-mesodermal vesicles became detached from the underlying endoderm during the processing procedures. In these cases the haemoglobin-containing cells were found to be associated with the underlying endoderm.

Comparing the results of the present experiment with those of the experiment in which the stage 8-9 pellucida ectoderm-plus-mesodermal fragments were cultured alone (Experiment 4.III, see Table 4.1), it is seen that the erythropoietic potential of the stage 8-9 pellucida ectoderm-plus-mesodermal fragments is indeed enhanced when they are cultured in the presence of the stage 4 opaca endoderm, compared to when they are

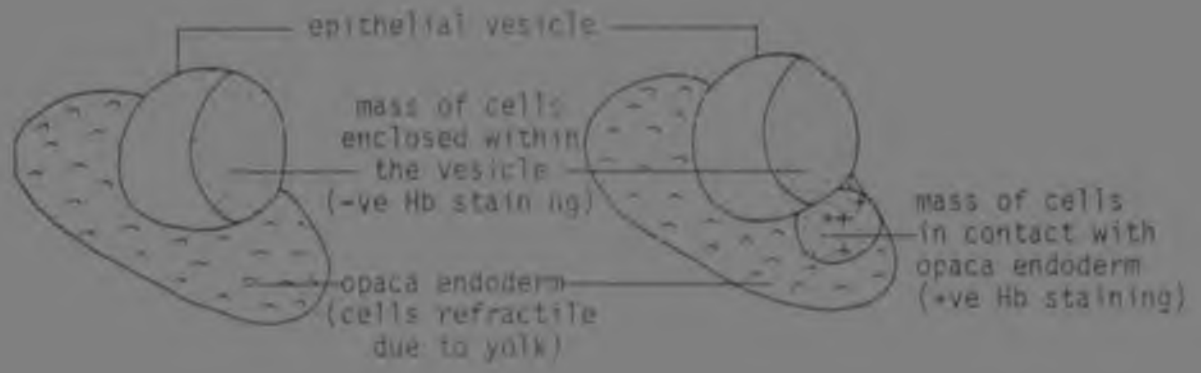


Figure 4.10

Diagrams illustrating the appearance, after 72 hours of culture, of stage 8-9 pellucida ectoderm-plus-mesodermal fragments in combination with stage 4 opaca endodermal fragments. The opaca endodermal tissue, identifiable owing to the refractile nature of its yolk-filled cells, tended to form a cellular layer, like a carpet, over the surface of the coverslip. The pellucida ectoderm-plus-mesodermal fragment is seen to have formed an epithelial vesicle enclosing a mass of cells in its interior. The diagram on the left illustrates the situation which occurred in the majority of the explants (64,7%), in which the epithelial vesicle appeared to enclose completely the inner mass of cells, separating these cells physically from the underlying endoderm. In all these cases the staining for haemoglobin was negative. The diagram on the right illustrates the situation which occurred in the remainder of the explants (35,3%), in which the epithelial vesicle did not enclose completely the inner mass of cells. In these explants, clumps of cells were also found on the outside of the vesicles, in contact with the underlying endoderm. Haemoglobin synthesis was seen to occur in a small number of the cells in this tissue which was in contact with the endoderm.

cultured alone (p = less than 0,01).

Simultaneously with the present two experiments (Experiments 4.V and 4.VI), a control experiment was carried out to verify that the isolated stage 4 opaca endoderm itself was not giving rise to blood cells (i.e., was not contaminated with erythropoietic cells).

Experiment 4.VII (endo (AOV, st 4))
(Control)

Wilt (1965a) and Miura and Wilt (1969) had previously found that the stage 4 opaca endoderm can be peeled cleanly from the overlying ectoderm and mesoderm, without any contaminating mesodermal cells adhering to it. This was re-checked and confirmed in the present study. As shown in Table 4.1, when stage 4 opaca endodermal fragments (Fig. 4.11) were isolated and cultured alone in small drops of medium for 72 hours, 100% of the explants (21 cases out of 21) exhibited negative staining for haemoglobin.

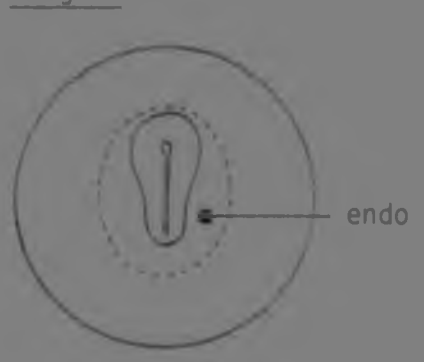


Figure 4.11

Diagram illustrating the area from where stage 4 opaca endodermal fragments were isolated.

4.3.1.2 Summary of the investigation of the erythropoietic potential of the stage 8-9 pellucida mesodermal tissue

The present study has investigated the erythropoietic potential of the stage 8-9 pellucida mesoderm when cultured alone or in combination with the other germinal layers from the AP and AOV. It was found that when the stage 8-9 pellucida mesoderm was cultured in its normal combination with the stage 8-9 pellucida ectoderm-plus-endoderm (Experiment 4.IV) or in combination with the stage 8-9 pellucida ectoderm only (Experiment 4.III) it did not differentiate erythropoietically. This finding agrees with the situation which occurs during normal development, in which the mesoderm of the stage 8-9 AP does not give rise to blood cells (see Chapters Two and Three). When, however, the stage 8-9 pellucida mesoderm was isolated from the pellucida ectoderm and endoderm and

(Text continues on page 187).

Table 4.1 Table summarizing the results of the study to investigate the ability of the stage 8-9 pellucida mesodermal fragments to give rise to blood cells when cultured alone or in combination with the other germinal layers from the AP and AOV (Section 4.3.1).

Experiments	Types of Explants	Total No. of Explants	No. of Explants (Categories of Haemoglobin Staining (See Key, Fig. 4.1 page 171))					% of Total No.				
			–	-+	+	++	+++	–	-+	+	++	+++
Expt. 4.I (Routine Control)	ecto + meso + endo (AOV, st 8-9)	167	0	0	0	0	167	0,0	0,0	0,0	0,0	100,0
Expt. 4.II	meso (AP, st 8-9)	77	67	6	4	0	0	87,0	7,8	5,1	0,0	0,0
Expt. 4.III	ecto + meso (AP, st 8-9)	20	18	2	0	0	0	90,0	10,0	0,0	0,0	0,0
Expt. 4.IV	ecto + meso + endo (AP, st 8-9)	72	64	4	4	0	0	88,9	5,6	5,6	0,0	0,0
Expt. 4.V	meso (AP, st 8-9) plus endo (AOV, st 4)	27	8	1	4	12	0	29,6	11,1	14,8	44,4	0,0
Expt 4.VI	ecto + meso (AP, st 8-9) plus endo (AOV, st 4)	17	11	0	1	5	0	64,7	0,0	5,9	29,4	0,0
Expt. 4 VII (Control)	endo (AOV, st 4)	21	21	0	0	0	0	100,0	0,0	0,0	0,0	0,0

Table 4.1 Table summarizing the results of the study to investigate the ability of the stage 8-9 pellucida mesodermal fragments to give rise to blood cells when cultured alone or in combination with the other germinal layers from the AP and AOV (Section 4.3.1).

Experiments	Types of Explants	Total No. of Explants	Categories of Haemoglobin Staining (See Key, Fig. 4.1 page 121). No. of Explants					% of Total No.				
			-	-+	+	++	+++	-	-+	+	++	+++
Expt. 4.I (Routine Control)	ecto + meso + endo (AOV, st 8-9)	167	0	0	0	0	167	0,0	0,0	0,0	0,0	100,0
Expt. 4.II	meso (AP, st 8-9)	77	67	6	4	0	0	87,0	7,8	5,1	0,0	0,0
Expt. 4.III	ecto + meso (AP, st 8-9)	20	18	2	0	0	0	90,0	10,0	0,0	0,0	0,0
Expt. 4.IV	ecto + meso + endo (AP, st 8-9)	72	64	4	4	0	0	88,9	5,6	5,6	0,0	0,0
Expt. 4.V	meso (AP, st 8-9) plus endo (AOV, st 4)	27	8	3	4	12	0	29,6	11,1	14,8	44,4	0,0
Expt. 4.VI	ecto + meso (AP, st 8-9) plus endo (AOV, st 4)	17	11	0	1	5	0	64,7	0,0	5,9	29,4	0,0
Expt. 4.VII (Control)	endo (AOV, st 4)	21	21	0	0	0	0	100,0	0,0	0,0	0,0	0,0

was cultured alone, there was suggestive evidence that some of its cells may have exhibited some potential for an erythropoietic differentiation (Experiment 4.II).

On the other hand, when the stage 8-9 pellucida mesoderm, or the stage 8-9 pellucida ectoderm-plus-mesoderm, was cultured in combination with stage 4 endodermal fragments, there was a significant enhancement in their erythropoietic differentiation (Experiments 4.V and 4.VI). This observation supports the finding from the mesodermal transplantation experiments of the previous chapter that some of the normally non-erythropoietic stage 8 pellucida mesodermal cells may differentiate erythropoietically if they are heterotopically transplanted into the AOV of stage 4-5 host blastoderms (Experiment 3.I, Chapter Three). During normal development, however, the stage 8-9 pellucida mesodermal cells remain in the AP, and therefore do not come into contact with the endoderm of the AOV (see Chapters Two and Three).

The results of the present study demonstrate that some of the stage 8-9 pellucida mesodermal cells are still pluripotent, and that their developmental pathways can still be influenced by environmental factors.

4.3.2 An investigation of the effects of the stage 4 opaca endoderm and ectoderm on the erythropoietic differentiation of the stage 4 opaca mesoderm - (Experiments 4.VIII to 4.XI)

In the studies described above in which stage 8-9 pellucida mesodermal fragments have been heterotopically transplanted into the stage 4 AOV (Section 3.3.1) or cultured in combination with the stage 4 opaca endoderm (Section 4.3.1), it has been found that the conditions in the stage 4 AOV, or indeed, the opaca endoderm itself, may influence the pathways of differentiation of some of the stage 8-9 pellucida mesodermal cells, causing them to differentiate along erythropoietic lines. The stage 4 opaca endoderm thus appears to provide an environment which stimulates mesodermal erythropoiesis.

These findings are in agreement with the observations of Wilt (1965a) and Miura and Wilt (1969). These workers cultured stage 4 opaca ectoderm-plus-mesodermal fragments either alone, or in the presence of stage 4 opaca endodermal fragments, and reported a qualitative and quantitative increase in the erythropoietic differentiation of the ectoderm-plus-

mesodermal explants when they were cultured in the presence of the endoderm, compared to when they were cultured alone.

In the present study four coverslip culture experiments have been carried out to investigate further the effects of the opaca tissues on mesodermal erythropoiesis. In these experiments, like those of Wilt (1965a) and Miura and Wilt (1969), attention has been focused on the erythropoietic potential of the stage 4 opaca mesoderm. However, the present experiments have extended the previous studies in that they are concentrated not only on the effects of the opaca endoderm on mesodermal erythropoiesis, but also on the question of whether the opaca ectoderm might exert any effect on mesodermal erythropoiesis.

A list of the test cultures performed in the present study is as follows:

Expt 4.VIII : ecto + meso (AOV, st 4) + endo (AOV, st 4);
Expt 4.IX : ecto + meso (AOV, st 4);
Expt 4.X : meso (AOV, st 4);
Expt 4.XI : meso (AOV, st 4) + endo (AOV, st 4);
Expt 4.VII : endo (AOV, st 4) = control.

The first two experiments in this study (Experiments 4.VIII and 4.IX) have been carried out along the lines of the experiments of Wilt (1965a) and Miura and Wilt (1969), and they confirm the findings of these authors that the stage 4 opaca endodermal fragments exert a stimulatory effect on the erythropoietic differentiation of the stage 4 opaca ectoderm-plus-mesodermal explants. This study is extended in the next two experiments in which the erythropoietic differentiation of isolated stage 4 opaca mesodermal fragments cultured alone (Experiment 4.X) or in combination with stage 4 opaca endodermal fragments (Experiment 4.XI) has been investigated. A comparison of the results of these two pairs of experiments indicates not only that the opaca endoderm exerts a stimulatory effect on mesodermal erythropoiesis, but furthermore, that the opaca ectoderm exerts an inhibitory effect on mesodermal erythropoiesis. This latter finding, that the opaca ectoderm exerts an inhibitory effect on mesodermal erythropoiesis, has not previously been recognized, and its significance will be dealt with in the discussion.

An outline of the experiments performed and the results obtained is presented below.

4.3.2.1 Culture experiments

Experiment 4.VIII (ecto + meso (AOV, st 4) plus)
(endo (AOV, st 4))

Explants composed of ectoderm-plus-mesoderm were isolated from the posterio-lateral region of the stage 4 AOV and recombined in culture with stage 4 opaca endodermal fragments isolated from the same region (Fig. 4.12).



Figure 4.12

Diagram illustrating the area from where stage 4 opaca ectoderm-plus-mesodermal fragments and stage 4 opaca endodermal fragments were isolated for combined culture.

After 72 hours of culture in small drops of medium, the following results were obtained, as tabulated in Table 4.2.

++ 15,8% of the explants (3 cases out of 19) exhibited brown (i.e., haemoglobin) staining in a large number of cells;

+++ 84,2% of the explants (16 cases out of 19) exhibited very intense haemoglobin staining in a large number of cells.

The results of this experiment have been compared with the results of the next experiment (Experiment 4.IX) in which stage 4 opaca ectoderm-plus-mesodermal fragments were cultured in the absence of the stage 4 opaca endoderm.

Experiment 4.IX (ecto + meso (AOV, st 4))

Explants composed of ectoderm-plus-mesoderm were isolated from the posterio-lateral region of the stage 4 AOV as above, and were cultured in the absence of the stage 4 opaca endoderm (Fig. 4.13).

Stage 4

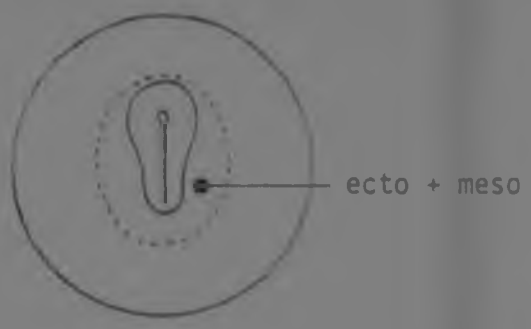


Figure 4.13

Diagram illustrating the area from where ectoderm-plus-mesodermal fragments were isolated from the AOV of stage 4 blastoderms.

After 72 hours of culture in small drops of medium, the following results were obtained, as tabulated in Table 4.2.

\- 35% of the explants (7 cases out of 20) stained the blue colour of the counterstain only (i.e., the staining for haemoglobin was negative);

-+ 30% of the explants (6 cases out of 20) exhibited traces of pale yellow staining (i.e., uncertain haemoglobin staining);

\+ 20% of the explants (4 cases out of 20) exhibited pale brown (i.e., pale haemoglobin) staining in a small number of cells;

++ 15% of the explants (3 cases out of 20) exhibited more intense haemoglobin staining in a small number of cells.

Comparing the results of this experiment with those of the previous experiment in which the stage 4 opaca ectoderm-plus-mesodermal fragments were cultured in combination with the stage 4 opaca endoderm (Experiment 4.VIII) (see Table 4.2), it is clear that there is indeed a qualitative and quantitative enhancement in the erythropoietic differentiation of the stage 4 opaca ectoderm-plus-mesodermal fragments when they are cultured in the presence of the stage 4 opaca endoderm (Experiment 4.VIII) compared to when they are cultured in its absence (Experiment 4.IX). The statistical comparison of these results indicates a highly significant difference between them (p = much less than 0,001). These results are in agreement with the findings of Wilt (1965a) and Miura and Wilt (1969).

Coupled with this observation it is found that when the stage 4 opaca ectoderm-plus-mesodermal fragments were cultured in the absence of the opaca endoderm, the explants tended to spread out and become flattened over the surface of the coverslips during the culturing period (Figs. 4.14, 4.15). This flattening tended to be more prominent in the explants in which the staining for haemoglobin was negative or uncertain (i.e., the explants in categories - and -+), in which cases the tissues tended to spread out and form a flattened carpet over the surface of the coverslips (Fig. 4.14, left hand micrograph). In the explants in which the staining for haemoglobin was more positive (i.e., the explants in categories + and ++), the ectoderm-plus-mesodermal fragments tended to be less flattened, although they still displayed the tendency to spread out over the surface of the coverslips (Fig. 4.14, right hand micrograph).



Figure 4.14

Photomicrographs of two stage 4 ectoderm-plus-mesodermal explants after 72 hours of culture, demonstrating the manner in which the explants tended to spread out over the surface of the coverslips during the culturing period. The left hand micrograph is an example of the situation which occurred in the 'negative' explants (categories - and -+), in which the explants tended to form a flattened carpet over the surface of the coverslips. The right hand micrograph is an example of the situation which occurred in the more 'positive' explants (categories + and ++), in which the explants tended to become less flattened, although they still displayed the tendency to spread out over the surface of the coverslips. (See histograms in Fig. 4.15).

x 177

(Text continues on page 193).

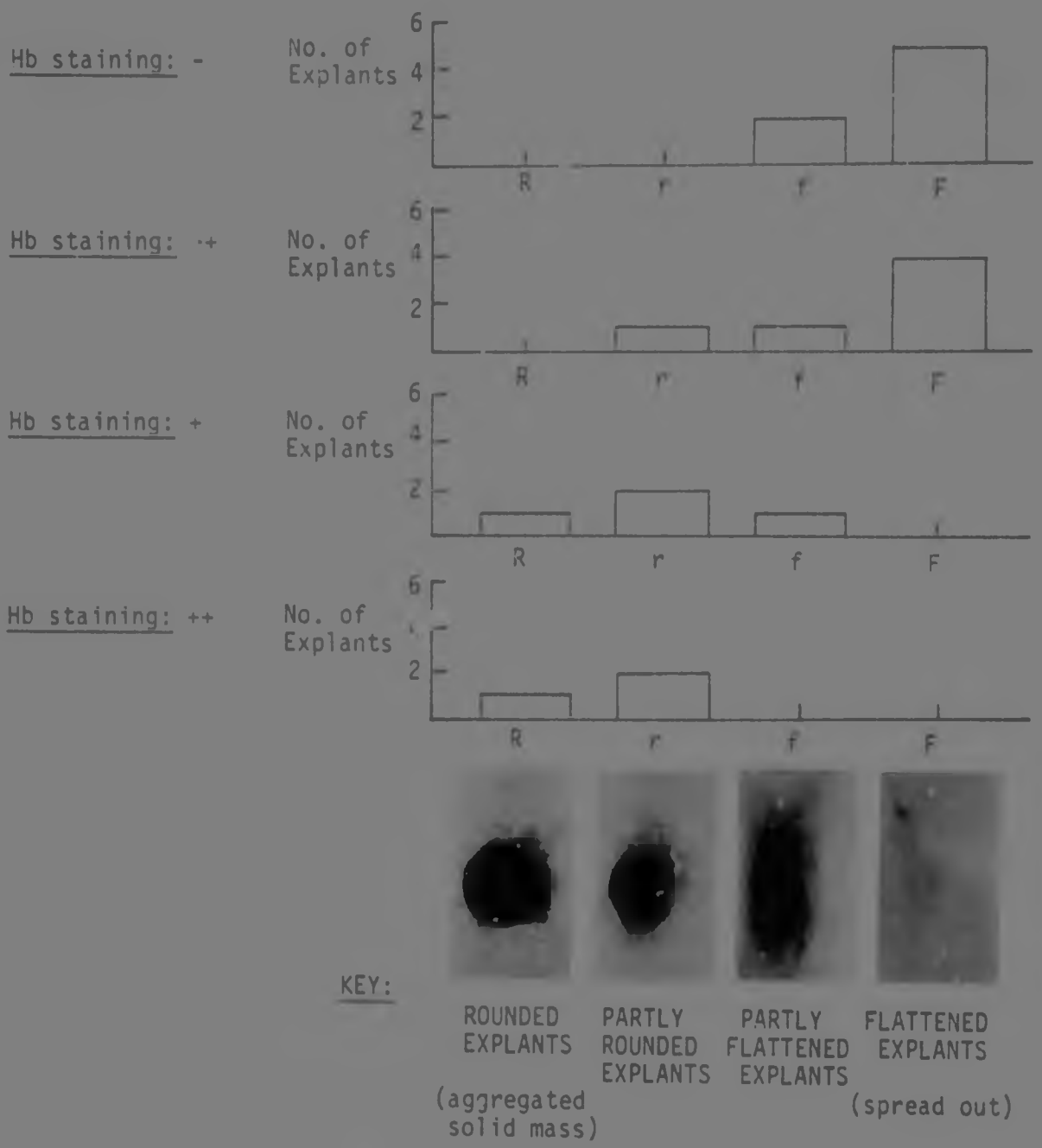


Figure 4.15

Histograms representing the results of Experiment 4.IX, in which stage 4 opaca ectoderm-plus-mesodermal fragments were cultured in the absence of the opaca endoderm. The results from each category of haemoglobin staining intensity have been plotted in a separate histogram. Each histogram represents the number of explants in a particular haemoglobin staining category plotted against the degree of tissue flattening. A key representing the increasing degrees of tissue spreading (flattening) is presented below the histograms. The results indicate the tendency for the explants in which the staining for haemoglobin is negative or uncertain (i.e., the explants in categories - and -+) to spread out and become flattened, whereas the explants in which the staining for haemoglobin is more positive (i.e., the explants in categories + and ++) tend to be less flattened.

key x 80

This trend is clear'y seen in a graphical representation of the results (Fig. 4.15), in which histograms have been drawn showing the number of explants in each of the four categories of haemoglobin staining intensity plotted against increasing degrees of tissue flattening. Each category of haemoglobin staining intensity has been plotted in a separate histogram. It is of interest to note that this type of flattening and spreading of the explants was not prevalent in the explants of the previous experiment in which the opaca ectoderm-plus-mesodermal fragments were recombined in culture with the opaca endoderm (Experiment 4.VIII). This would thus suggest that the endoderm may prevent the spreading of the ectoderm-plus-mesodermal tissue.

As outlined above, the present study has been extended by comparing the results of the above two experiments with the results of the following two experiments (Experiments 4.X and 4.XI) in which the erythropoietic potential of isolated stage 4 opaca mesodermal fragments has been studied. In these experiments stage 4 opaca mesodermal fragments have been cultured a . (Experiment 4.X) or have been recombined in culture with stage 4 opaca endodermal fragments (Experiment 4.XI).

Experiment 4.X (meso (AOV, st 4))

Stage 4 mesodermal fragments were isolated from the posteriolateral region of the AOV (Fig. 4.16) and cultured alone in small drops of medium.

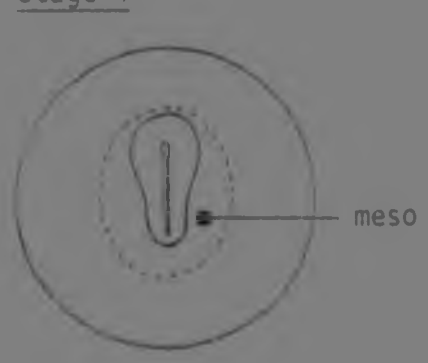


Figure 4.16

Diagram illustrating the area from where mesodermal fragments were isolated from the AOV of stage 4 b'.stoderms.

After 72 hours of culture, the following results were obtained, as tabulated in Table 4.2.

-+ 3,6% of the explants (1 case out of 28) exhibited traces of pale yellow staining (i.e., uncertain haemoglobin staining);

\+ 17,9% of the explants (5 cases out of 28) exhibited pale brown (i.e., pale haemoglobin) staining in a large number of cells;

++ 50% of the explants (14 cases out of 28) exhibited more intense haemoglobin staining in a large number of cells;

+++ 28,6% of the explants (8 cases out of 28) exhibited very intense haemoglobin staining in a large number of cells.

Comparing the results of this experiment with the results of the previous experiment (Experiment 4.IX) in which stage 4 opaca ectoderm-plus-mesodermal explants were cultured alone (see Table 4.2), it can be seen that the stage 4 opaca mesodermal fragments cultured alone (Experiment 4.X) produce qualitatively and quantitatively more haemoglobin than the stage 4 opaca ectoderm-plus-mesodermal fragments (Experiment 4.IX). Indeed, a statistical comparison of the results of these two experiments reveals a highly significant difference between them (p = much less than 0,001). Thus, when the stage 4 opaca mesoderm is cultured in the presence of the stage 4 opaca ectoderm it produces significantly less haemoglobin than it does when cultured in the absence of the opaca ectoderm. The opaca ectoderm therefore appears to exert an inhibitory influence on the erythropoietic differentiation of the mesodermal cells. An inhibitory effect of the opaca ectoderm on the erythropoietic differentiation of the opaca mesodermal cells has not previously been recognized.

Experiment 4.XI (meso (AOV, st 4) + endo (AOV, st 4))

In this experiment, stage 4 mesodermal fragments were isolated from the postero-lateral region of the AOV and recombined with stage 4 opaca endodermal fragments isolated from the same region (Fig. 4.17).

After 72 hours of culture in small drops of medium, the following results were obtained, as tabulated in Table 4.2.

\+ 4,5% of the explants (1 case out of 22) exhibited pale brown (i.e., pale haemoglobin) staining in a large number of cells;

++ 13,6% of the explants (3 cases out of 22) exhibited fairly intense haemoglobin staining in a large number of cells;

+++ 81,8% of the explants (18 cases out of 22) exhibited very intense haemoglobin staining in a large number of cells.

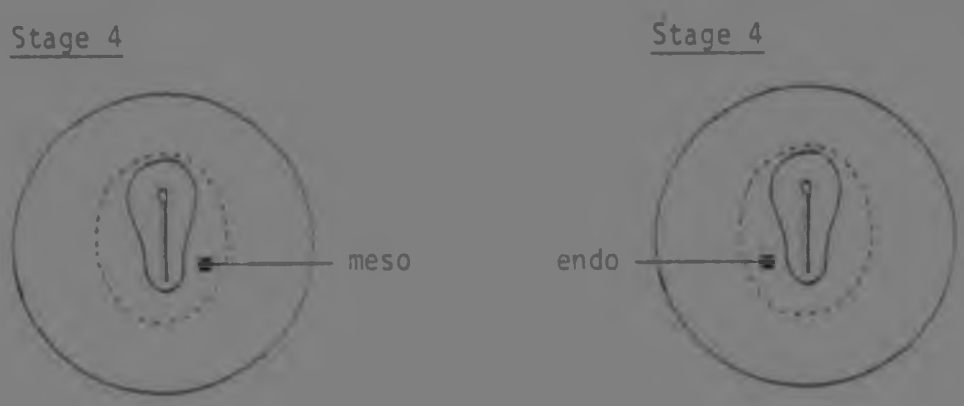


Figure 4.17

Diagram illustrating the area from where stage 4 opaca mesodermal fragments and stage 4 opaca endodermal fragments were isolated for combined culture.

The results of this experiment are significantly greater than the results of the previous experiment (Experiment 4.X) in which the stage 4 opaca mesodermal fragments were cultured alone (p = less than 0,001). Thus, it is once again evident that the stage 4 opaca endoderm exert, a stimulatory effect on the erythropoietic differentiation of the mesodermal cells.

Control cultures

The control experiment carried out to verify that the isolated opaca endodermal fragments were not contaminated with adhering mesodermal cells is described on page 135 (Experiment 4.VII). As tabulated in Table 4.2, when stage 4 opaca endodermal fragments were cultured alone in small drops of medium for 72 hours, 100 of the explants (21 cases out of 21) exhibited negative staining for haemoglobin.

Routine control cultures were also carried out during the course of all the above experiments to ensure that the culture medium and the o-dianisidine stain were always optimal (Experiment 4.I, page 173). As tabulated in Table 4.2, when explants composed of all three germinal layers were isolated from the posterio-lateral part of the stage 8-9 AOV (i.e., from a normally erythropoietic part of the blastoderm), 100% of the ex-

plants (167 cases out of 167) were found to be in category +++.

4.3.2.2 Summary of the investigation of the effects of the stage 4 opaca endoderm and ectoderm on the erythropoietic differentiation of the stage 4 opaca mesoderm

The present study has investigated the effects of the stage 4 opaca endoderm and ectoderm on the erythropoietic differentiation of the stage 4 opaca mesoderm. In agreement with the findings of Wilt (1965a) and Miura and Wilt (1969) it was found that the erythropoietic differentiation of stage 4 opaca ectoderm-plus-mesodermal explants was significantly enhanced when the explants were cultured in combination with the stage 4 opaca endoderm (Experiment 4.VIII) compared to when they were cultured alone (Experiment 4.IX). Likewise, it was found that the erythropoietic differentiation of isolated stage 4 opaca mesodermal explants was significantly enhanced when they were cultured in combination with the stage 4 opaca endoderm (Experiment 4.XI) compared to when they were cultured alone (Experiment 4.X). Both sets of experiments therefore confirm the view that the stage 4 opaca endoderm exerts a stimulatory effect on mesodermal erythropoiesis.

When, on the other hand, the erythropoietic differentiation of the isolated stage 4 opaca mesoderm was compared with that of the stage 4 opaca mesoderm cultured in combination with the stage 4 opaca ectoderm (compare Experiments 4.IX and 4.X), it was found that the stage 4 opaca mesoderm cultured alone produced significantly more haemoglobin than the stage 4 opaca mesoderm cultured in the presence of the stage 4 opaca ectoderm. This indicates that the stage 4 opaca ectoderm exerts an inhibitory effect on mesodermal erythropoiesis.

Coupled with this finding, it was found that the stage 4 opaca ectoderm-plus-mesodermal explants have a tendency to spread out and become flattened over the surface of the coverslips during the culturing period. This tendency to flatten was seen to be more prominent in those ectoderm-plus-mesodermal explants which exhibited negative or uncertain staining for haemoglobin, and less prominent in those explants which exhibited more positive staining for haemoglobin. This would suggest that the inhibitory effect of the opaca ectoderm on mesodermal erythropoiesis may be associated with the tendency of the ectoderm-plus-

(Text continues on page 198)

Table 4.2 Table summarizing the results of the study to investigate the effects of the stage 4 opaca embryoderm and ectoderm on the erythropoietic differentiation of the stage 4 opaca mesoderm (Section 4.1.2).

Experiments	Types of Explants	Total No. of Explants	Categories of Haemoglobin Staining (See Key, Fig. 4.1 page 171). No. of Explants					% of Total No				
			-	-+	+	++	+++	-	-+	+	++	+++
Expt. 4.VIII	ecto + meso (AOV, st 4) plus endo (AOV, st 4)	19	0	0	0	3	16	0,0	0 n	0,0	15,8	84,2
Expt. 4.IX	ecto + meso (AOV, st 4)	20	7	6	4	3	0	35,0	30,0	20,0	15,0	0,0
Expt. 4.X	meso (AOV, st 4)	28	0	1	5	14	8	0,0	3,6	17,9	50,0	28,6
Expt. 4.XI	meso (AOV, st 4) plus endo (AOV, st 4)	22	0	0	1	3	18	0,0	0,0	4,5	13,6	81,8
Expt. 4.VII (Control)	endo (AOV, st 4)	21	21	0	0	0	0	100,0	0,0	0,0	0,0	0,0
Expt. 4.I (Routine Control)	ecto + meso + endo (AOV, st 8-9)	167	0	0	0	0	167	0,0	0,0	0,0	0,0	100,0

mesodermal explants to become flattened. Significantly, it was found that when the stage 4 opaca ectoderm-plus-mesodermal fragments were combined in culture with the stage 4 opaca endoderm (Experiment 4.VIII), not only was there a significant enhancement in their erythropoietic differentiation, but in addition, the explants did not exhibit the strong tendency to flatten. The stage 4 opaca endoderm was seen to overcome the inhibitory effects of the opaca ectoderm on mesodermal erythropoiesis.

4.3.3 An investigation of the erythropoietic potential of the stage 4 pellucida mesoderm when cultured alone or in combination with the other germinal layers from the AP and AOV - (Experiments 4.XIi to 4.XV)

A central question being focused upon in this study of yolk sac vasculogenesis is the question of why the yolk sac mesodermal cells tend to give rise to intravascular blood cells in the AOV, but not in the AP. The question has already been posed as to whether the mesodermal cells in the AP and AOV are different from each other, or whether environmental factors in the AP and AOV play an important role in determining the pathways of differentiation of the mesodermal cells (see Sections 3.3, 4.3.1, and 4.3.2). In the present section attention has been focused on the erythropoietic potential of the stage 4 pellucida mesoderm.

Murray (1932) explanted fragments of the stage 4 primitive streak in culture and found that they gave rise to blood-islands ("haemangioblasts") and their differentiated derivatives *in vitro*. From this Murray concluded that ". . . the mesenchymal cells which form the haemangioblasts must be regarded, even while they are still in the primitive streak, as self-differentiating cells, predetermined, or at least strongly biassed, towards the formation of the group of cell types which are derived from the haemangioblasts." (Murray, 1932 page 515). This was later verified by Settle (1954) and Wilt (1974), who found that the determination of the potentially erythropoietic cells has already occurred by the time the egg is laid (i.e., by stage 1). This leads to the question of why the potentially erythropoietic mesodermal cells which migrate through the AP into the ACV do not aggregate in the AP and give rise to erythrocytes there, but rather continue to migrate centrifugally into the AOV where they then undergo their overt erythropoietic

differentiation.

In a previous section (Section 4.3.1) it has been shown that whereas the stage 8-9 pellucida mesoderm does not differentiate erythropoietically in the presence of the stage 8-9 pellucida tissues, it may exhibit some potential for erythropoiesis when it is isolated and cultured alone. This could be interpreted to suggest that the pellucida environment may exert an inhibitory effect on mesodermal erythropoiesis. It has been shown, furthermore, that when the stage 8-9 pellucida mesoderm is cultured in the presence of the stage 4 opaca endoderm, there is a significant enhancement in the erythropoietic differentiation of these cells.

In view of these findings, the experiments in the present section have been devised to investigate further the effects of the pellucida environment on mesodermal erythropoiesis. The question being investigated is whether the stage 4 pellucida tissues exert an inhibitory effect on the erythropoietic differentiation of the stage 4 pellucida mesoderm. Since the stage 4 pellucida mesoderm constitutes a migratory population of cells which will subsequently enter into the AOV and differentiate erythropoietically (see Chapter Two), a comparison has been made of the extent of erythropoiesis undergone by the stage 4 pellucida mesoderm when it is cultured in combination with the pellucida or opaca tissues.

The following test cultures have been performed, the details of which are outlined below:

Expt 4.XII : meso (AP, st 4);
Expt 4.XIII : ecto + meso (AP, st 4);
Expt 4.XIV : ecto + meso + endo (AP, st 4);
Expt 4.XV : meso (AP, st 4) + endo (AOV, st 4);
Expt 4.VII : endo (AOV, st 4) = control.

4.3.3.1 Culture experiments

Experiment 4.XII (meso (AP, st 4))

In this experiment the erythropoietic potential of isolated stage 4 pellucida mesodermal fragments was investigated. 0,5mm x 0,5mm mesodermal fragments were isolated from the AP of stage 4 blastoderms (Fig. 4.18) and cultured alone in small drops of medium.

Stage 4

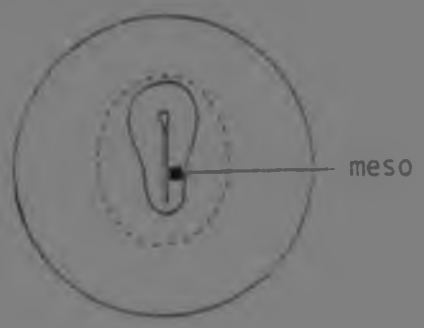


Figure 4.18

Diagram illustrating the area from where mesodermal fragments were isolated from the AP of stage 4 blastoderms.

After 72 hours of culture, the following results were obtained, as tabulated in Table 4.3.

-+ 18,2% of the explants (4 cases out of 22) exhibited traces of pale yellow staining (i.e., uncertain haemoglobin staining);

+ 31,8% of the explants (7 cases out of 22) exhibited pale brown (i.e., pale haemoglobin) staining in a large number of cells;

++ 36,4% of the explants (8 cases out of 22) exhibited more intense haemoglobin staining in a large number of cells;

+++ 13,6% of the explants (3 cases out of 22) exhibited very intense haemoglobin staining in a large number of cells.

For comparison, the next experiment was carried out in which the stage 4 pellucida mesoderm was cultured in combination with its own pellucida ectoderm (Experiment 4.XIII).

Experiment 4.XIII (ecto + meso (AP, st 4))

Stage 4 pellucida ectoderm-plus-mesodermal fragments were isolated from the same region of the blastoderm as before (Fig. 4.19), and cultured in small drops of medium.

After 72 hours of culture, the following results were obtained, as tabulated in Table 4.3.

- 30,4% of the explants (7 cases out of 23) stained the blue colour of the counterstain only (i.e., the staining for haemoglobin was negative);

-+ 17,4% of the explants (4 cases out of 23) exhibited traces of pale yellow staining (i.e., uncertain haemoglobin staining);

+ 21,8% of the explants (5 cases out of 23) exhibited pale brown (i.e., pale haemoglobin) staining in a small number of cells;

++ 30,4% of the explants (7 cases out of 23) exhibited more intense haemoglobin staining in a variable number of cells.

Stage 4

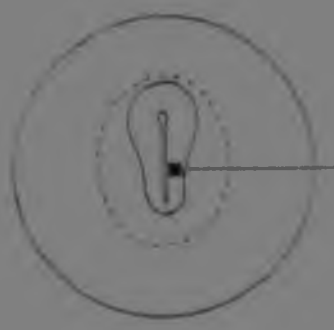


Figure 4.19

Diagram illustrating the area from where ectoderm-plus-mesodermal fragments were isolated from the AP of stage 4 blastoderms.

Comparing the results of this experiment with those of the previous experiment in which stage 4 pellucida mesodermal fragments were cultured alone (Experiment 4.XII), it is seen that the stage 4 pellucida mesoderm cultured alone produces qualitatively and quantitatively more haemoglobin than the stage 4 pellucida mesoderm cultured in combination with its own pellucida ectoderm (see Table 4.3). The difference between these results is significant (p = less than 0,01). Thus, the stage 4 pellucida ectoderm, like the stage 4 opaca ectoderm (Section 4.3.2) appears to exert an inhibitory influence on the erythropoietic differentiation of the mesodermal cells.

A point of relevance which arose in the present experiment is that many of the stage 4 pellucida ectoderm-plus-mesodermal explants tended to spread out and become flattened over the surface of the coverslips

during the culturing period. This situation is analogous to that which occurred when the stage 4 opaca ectoderm-plus-mesodermal explants were cultured (Experiment 4.IX, see Figs. 4.14 and 4.15). As found in Experiment 4.IX, the flattening of the ectoderm-plus-mesodermal explants tended to be more prominent in the explants in which the staining for haemoglobin was negative or uncertain (i.e., the explants in categories - and -+) in which cases the explants tended to spread out and form a flattened carpet on the surface of the coverslips (cf. Fig. 4.14, left hand micrograph). However, in three of the 'negative' cases the explants, rather than flattening, tended to form balloon-like epithelial vesicles which, by focusing the microscope at different planes, were seen to enclose a clump of cells in their interior. This situation is analogous to that which occurred when the stage 8-9 pellucida ectoderm-plus-mesodermal explants were cultured (Experiment 4.III, see Fig. 4.6).

In the more 'positive' categories (i.e., the explants in categories + and ++) the tissue tended to be less flattened, although it still displayed the tendency to spread out over the surface of the coverslips. This situation is again analogous with that which occurred in Experiment 4.IX, in which the explants in the more 'positive' categories were likewise seen to be less flattened (see Fig. 4.14, right hand micrograph).

This trend is clearly seen in Fig. 4.20, in which the results of the present experiment have been presented in the form of histograms. As done for Experiment 4.IX (see Fig. 4.15), a separate histogram has been plotted for each category of haemoglobin staining intensity. Each histogram represents the number of explants plotted against increasing degrees of tissue flattening. The histograms in Fig. 4.20 also show the three cases in which the explants tended to form balloon-like epithelial vesicles.

For comparison, the next experiment was carried out in which stage 4 pellucida fragments composed of all three germinal layers were cultured (Experiment 4.XIV).

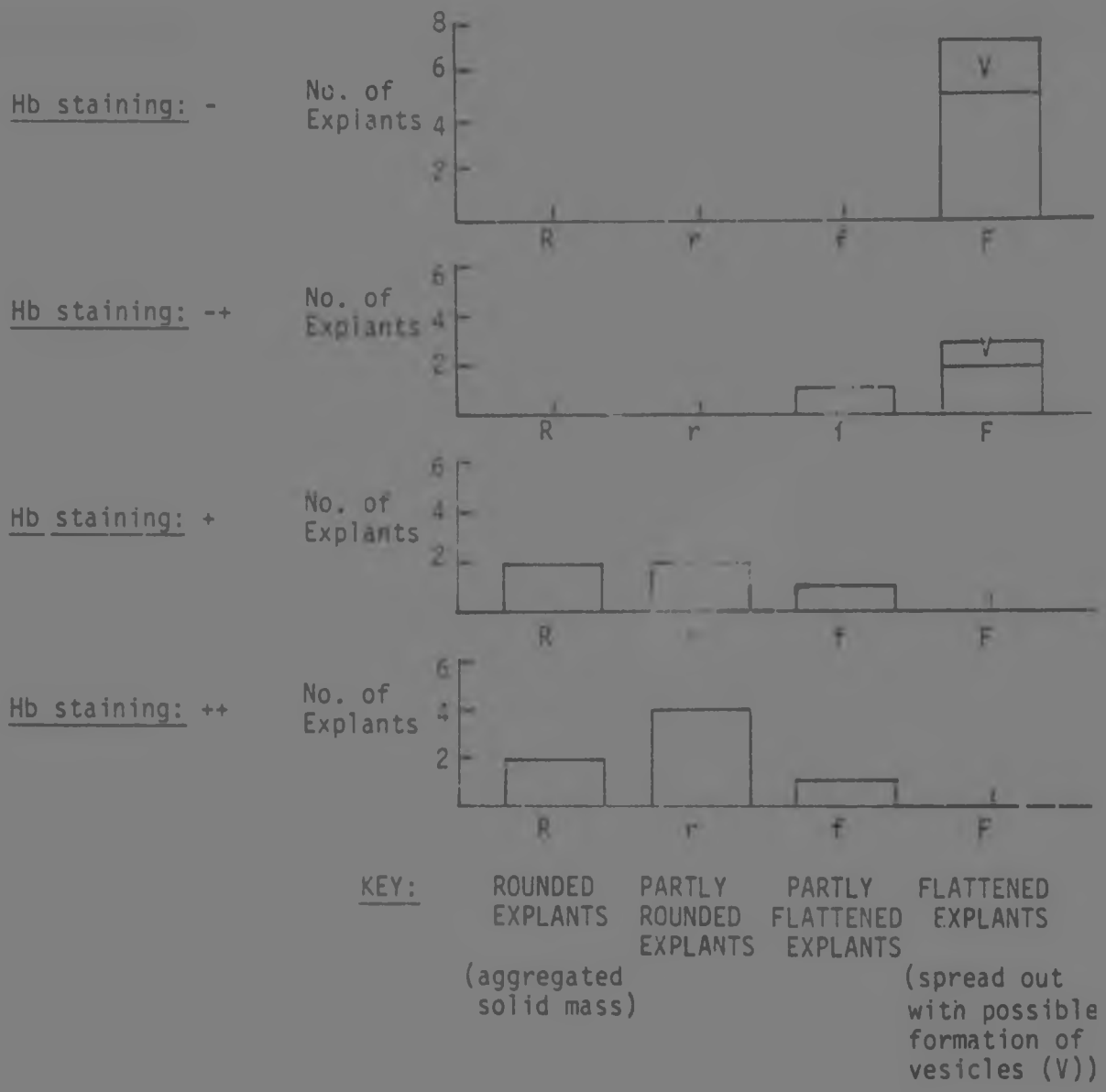


Figure 4.20

Histograms representing the results of Experiment 4.XIII, in which stage 4 pellucida ectoderm-plus-mesodermal fragments were cultured. The results from each category of haemoglobin staining intensity have been plotted in a separate histogram. Each histogram represents the number of explants in a particular haemoglobin staining category plotted against the degree of tissue flattening. Also included in the histograms are the three cases in which the explants were seen to have formed balloon-like epithelial vesicles which enclosed a mass of cells in their interiors. The results indicate the tendency for the explants in which the staining for haemoglobin is negative or uncertain (i.e., the explants in categories - and -+) to be flattened or to form balloon-like vesicles, whereas the explants in which the staining for haemoglobin is more positive (i.e., the explants in categories + and ++) tend to be less flattened. The key representing the increasing degrees of tissue spreading (flattening) is the same as that presented below the histograms of Fig. 4.15.

Experiment 4.XIV (ecto + meso + endo (AP, st 4))

0,5mm x 0,5mm fragments composed of all three germinal layers (i.e., ectoderm, mesoderm and endoderm) were isolated from the same region of the stage 4 AP (Fig. 4.21), and cultured in small drops of medium.

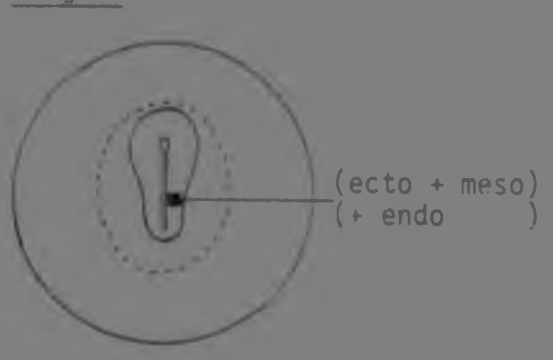


Figure 4.21

Diagram illustrating the area from where fragments composed of all three germinal layers (i.e., ectoderm, mesoderm and endoderm) were isolated from the AP of stage 4 blastoderms.

After 72 hours of culture, the following results were obtained, as tabulated in Table 4.3.

- 15,8% of the explants (3 cases out of 19) stained the blue colour of the counterstain only (i.e., the staining for haemoglobin was negative);
-+ [illegible] of the explants (3 cases out of 19) exhibited traces of yellow staining (i.e., uncertain haemoglobin staining);
+ 36,8% of the explants (7 cases out of 19) exhibited pale brown (i.e., pale haemoglobin) staining in a large number of cells;
++ 31,6[illegible] of the explants (6 cases out of 19) exhibited more intense haemoglobin staining in a variable number of cells.

Although the erythropoietic differentiation of the explants in the present experiment appears to be slightly higher than that of the explants in the previous experiment in which the stage 4 pellucida ectoderm-plus-mesodermal fragments were cultured in the absence of the pellucida endoderm (Experiment 4.XIII), nevertheless the difference

between them is not statistically significant (p = 0,5). Coupled with this observation it is found that the explants in the present experiment also exhibited the tendency to spread out and become flattened over the surface of the coverslips, and that this flattening is likewise more prominent in the explants in which the staining for haemoglobin was negative or uncertain (cf. Fig. 4.20). This suggests that the negative influence exerted by the pellucida ectoderm on mesodermal erythropoiesis is not overcome by the presence of the stage 4 pellucida endoderm. Indeed, the results of the present experiment in which all three germinal layers of the stage 4 AP were cultured together are found to be less positive than the results of the experiment in which the stage 4 pellucida mesoderm was cultured alone (Experiment 4.XII - see Table 4.3), and the difference is statistically significant (p = 0,05). Thus the environment of the AP as a whole does indeed appear to exert a negative influence on mesodermal erythropoiesis.

For comparison, the next experiment was carried out in which the stage 4 pellucida mesoderm was cultured in the presence of the stage 4 opaca endoderm (Experiment 4.XV). The aim of this experiment was to determine the extent of the erythropoietic potential of the stage 4 pellucida mesoderm when it was cultured in the presence of a proven erythropoietic stimulator.

Experiment 4.XV (meso (AP, st 4) + endo (AOV, st 4))

Stage 4 pellucida mesoderm was isolated and cultured in combination with stage 4 opaca endoderm (Fig. 4.22).



Figure 4.22

Diagram illustrating the areas of the stage 4 blastoderm from where pellucida mesodermal fragments and opaca endodermal fragments were isolated for combined culture.

After 72 hours of culture in small drops of medium, the following results were obtained, as tabulated in Table 4.3.

\+ 5% of the explants (1 case out of 20) exhibited pale brown (i.e., pale haemoglobin) staining in a large number of cells;

++ 20% of the explants (4 cases out of 20) exhibited more intense haemoblogin staining in a large number of cells;

+++ 75% of the explants (15 cases out of 20) exhibited very intense haemoglobin staining in a large number of cells.

The results of this experiment show a significant enhancement in the erythropoietic differentiation of the stage 4 pellucida mesodermal cells compared with that in the previous three experiments in which the stage 4 pellucida mesoderm was cultured either alone (Experiment 4.XII), or in combination with its own pellucida ectoderm (Experiment 4.XIII), or in combination with its own pellucida ectoderm-plus-endoderm (Experiment 4.XIV) (p = much less than 0,001 for all three comparisons). Moreover, when the erythropoietic differentiation of the present explants (i.e., the stage 4 pellucida mesoderm cultured in combination with the stage 4 opaca endoderm) is compared with that of the explants in which the stage 4 opaca mesoderm was cultured in combination with the stage 4 opaca endoderm (Experiment 4.XI), it is found that there is not a significant difference between the erythropoietic differentiation of the pellucida and opaca mesodermal cells when they are cultured in the presence of the stage 4 opaca endoderm (p = greater than 0,5).

It would thus appear that the stage 4 pellucida mesoderm is unable to realize its full erythropoietic potential when subjected to the environmental conditions of the AP, i.e., the AP appears to be unfavourable for mesodermal erythropoiesis. However, when these mesodermal cells come into contact with the opaca endodermal cells, they are stimulated to proceed with their erythropoietic differentiation. This contact between the mesoderm and the opaca endoderm would occur during normal development when the stage 4 pellucida mesodermal cells eventually migrated into the AOV itself (see Chapter Two).

Control cultures

The control experiment carried out to verify that the isolated stage 4 opaca endodermal fragments were not contaminated with adhering

(Text continues on page 208).

Table 4.3 Table summarizing the results of the study to investigate the erythropoietic potential of the stage 4 pellucida mesoderm when cultured alone or in combination with the other germinal layers from the AP and AOV (Section 4.3.3).

Experiments	Types of Explants	Total No. of Explants	No. of Explants (Categories of Haemoglobin Staining (See Key, Fig. 4.1 page 171))					% of Total No.				
			-	-+	+	++	+++	-	-+	+	++	+++
Expt. 4.XII	meso (AP, st 4)	22	0	4	7	8	3	0,0	18,2	31,8	36,4	13,6
Expt. 4.XIII	ecto + meso (AP, st 4)	23	7	4	5	7	0	30,4	17,4	21,8	30,4	0,0
Expt. 4.XIV	ecto + meso + endo (AP, st 4)	19	3	3	7	6	0	15,8	15,8	36,8	31,6	0,0
Expt. 4.XV	meso (AP, st 4) plus endo (AOV, st 4)	20	0	0	1	4	15	0,0	0,0	5,0	20,0	75,0
Expt. 4.VII (Control)	endo (AOV, st 4)	21	21	0	0	0	0	100,0	0,0	0,0	0,0	0,0
Expt. 4.I (Routine Control)	ecto + meso + endo (AOV, st 3-4)	167	0	0	0	0	167	0,0	0,0	0,0	0,0	100,0

mesodermal cells has been described on page 185 (Experiment 4.VII). As tabulated in Table 4.3, when stage 4 opaca endodermal fragments were cultured alone in small drops of medium for 72 hours, 100% of the explants (21 cases out of 21) exhibited negative staining for haemoglobin.

Routine control cultures were also carried out during the course of all the above experiments to ensure that the culture medium and the o-dianisidine stain were always optimal (Experiment 4.I, page 173). As tabulated in Table 4.3, when explants composed of all three germinal layers were isolated from a normally erythropoietic part of the blastoderm, 100% of the explants (167 cases out of 167) exhibited very intense staining for haemoglobin.

4.3.3.2 Summary of the investigation of the erythropoietic potential of the stage 4 pellucida mesoderm

The present study has compared the erythropoietic differentiation of the stage 4 pellucida mesoderm when cultured alone or in combination with the ectoderm and/or endoderm of the AP and AOV. It was found that the erythropoietic differentiation of stage 4 pellucida ectoderm-plus-mesodermal explants (Experiment 4.XIII) was significantly lower than that of the isolated stage 4 pellucida mesodermal fragments cultured alone (Experiment 4.XII). (This situation is analogous to the finding that the erythropoietic differentiation of stage 4 opaca ectoderm-plus-mesodermal explants was lower than that of the stage 4 opaca mesoderm cultured alone (Experiments 4.IX and 4.X, Section 4.3.2)). This indicates that the stage 4 pellucida ectoderm, like the stage 4 opaca ectoderm, exerts an inhibitory effect on mesodermal erythropoiesis. Again, the inhibitory effect appears to be coupled with the tendency of the ectoderm-plus-mesodermal explants to spread out and become flattened over the surface of the coverslips, or with the tendency of the explants to form balloon-like epithelial vesicles enclosing a mass of cells. It was found, however, that this inhibitory effect of the stage 4 pellucida ectoderm on mesodermal erythropoiesis was not overcome by the presence of the stage 4 pellucida endoderm, since the erythropoietic differentiation of the stage 4 pellucida ectoderm-plus-mesodermal explants (Experiment 4.XIII) was not significantly different from that of the stage 4 pellucida explants composed of all three germinal layers (i.e., ectoderm, mesoderm and endoderm) (Experiment 4.XIV).

Coupled with this, these stage 4 pellucida explants which contained the pellucida endoderm still exhibited the tendency to spread out and flatten, or to form balloon-like vesicles. It thus appears that the environment of the stage 4 AP as a whole exerts a negative influence on mesodermal erythropoiesis. (This situation differs from the finding that the stage 4 opaca endoderm can totally overcome the inhibitory effect of the stage 4 opaca ectoderm on mesodermal erythropoiesis (Experiments 4.VIII and 4.IX, Section 4.3.2)).

When, on the other hand, the stage 4 pellucida mesoderm was cultured in combination with the stage 4 opaca endoderm, there was a significant enhancement in the erythropoietic differentiation of the mesodermal cells (Experiment 4.XV). Indeed, the erythropoietic differentiation of the stage 4 pellucida mesoderm in the presence of the stage 4 opaca endoderm (Experiment 4.XV) was very comparable with that of the stage 4 opaca mesoderm in the presence of the stage 4 opaca endoderm (Experiment 4.XI).

The results of the present study thus indicate that the stage 4 pellucida ectoderm and endoderm comprise an environment which is unfavourable for mesodermal erythropoiesis, in that the stage 4 pellucida mesoderm is not stimulated to realize its full erythropoietic potential in the AP. On the other hand, when the stage 4 pellucida mesodermal cells come into contact with the stage 4 opaca endoderm they are stimulated to realize their full erythropoietic potential. This would occur during normal development when the stage 4 pellucida mesodermal cells eventually migrated into the AOV itself.

4.3.4 An investigation of the effects of the stage 1-2 opaca ectoderm and endoderm on mesodermal erythropoiesis

A key observation consistently made in each of the three previous sections in this chapter (Sections 4.3.1, 4.3.2 and 4.3.3) is that the stage 4 opaca endoderm exerts a stimulatory influence on the erythropoietic differentiation of the mesodermal cells. This observation is in concordance with the findings of Wilt (1965a) and Miura and Wilt (1969).

On the other hand, Bennett (1973), using the enzyme cysteine lyase

as a marker of endodermal cell differentiation, found that the differentiation of the opaca endoderm itself is dependent upon the presence of the yolk sac mesoderm, and is temporally coupled with the migration of the mesoderm into the AOV. The question then arises as to whether the stimulatory influence of the opaca endoderm on mesodermal erythropoiesis is a result of the differentiation of the opaca endoderm itself. Phrased in another way, the question may be posed as to whether the opaca endoderm of younger blastoderms (in which the mesoderm has not yet entered into the AOV) is as capable of stimulating mesodermal erythropoiesis as that of older blastoderms (in which contact between the mesoderm and the opaca endoderm has already taken place).

In the following experiments the effect of the stage 1-2 opaca endoderm and ectoderm on mesodermal erythropoiesis has therefore been investigated:

Expt 4.XVI : meso (AP, st 4) + endo (AOV, st 1-2);
Expt 4.XVII : meso (AP, st 8-9) + endo (AOV, st 1-2);
Expt 4.XVIII : meso (AP, st 4) + ecto + endo (AOV, st 1-2);
Expt 4.XIX : meso (AP, st 8-9) + ecto + endo (AOV, st 1-2);
Expt 4.XX : endo (AOV, st 1-2) = control;
Expt 4.XXI : ecto + endo (AOV, st 1-2) = control.

(Note: For convenience. the hypoblast and epiblast of the stage 1-2 blastoderm are referred to as endoderm and ectoderm respectively).

An outline of the experiments performed and the results obtained is presented below.

4.3.4.1 Culture experiments

Experiment 4.XVI (meso (AP, st 4) + endo (AOV, st 1-2))

Stage 4 pellucida mesodermal fragments were isolated and combined on coverslips with stage 1-2 opaca endodermal fragments (Fig. 4.23). Stage 4 pellucida mesoderm was chosen in preference to stage 4 opaca mesoderm because the latter has already come into contact with the stage 4 opaca endodermal cells, and thus may have already been influenced by the opaca endoderm.

After 72 hours of culture in small drops of medium, the following results were obtained, as tabulated in Table 4.4.

\+ 30% of the explants (3 cases out of 10) exhibited pale brown (i.e., pale haemoglobin) staining in a large number of cells;

++ 40% of the explants (4 cases out of 10) exhibited more intense haemoglobin staining in a large number of cells;

+++ 30% of the explants (3 cases out of 10) exhibited very intense haemoglobir taining in a large number of cells.



Figure 4.23

Diagram illustrating the areas from where stage 4 pellucida mesodermal fragments and stage 1-2 opaca endodermal fragments were isolated for combined culture.

Comparing the results of this experiment with the results of the experiment in which stage 4 pellucida mesodermal fragments were cultured alone (Experiment 4.XII, Section 4.3.3), it is found that there is no significant difference between the erythropoietic differentiation of the mesodermal cells when they are cultured in the presence of the stage 1-2 opaca endoderm or when they are cultured alone (p = greater than 0,1). Thus, the stage 1-2 opaca endoderm does not appear to affect the erythropoietic differentiation of the stage 4 pellucida mesodermal cells.

This situation is in great contrast to the finding that when the stage 4 pellucida mesoderm is cultured in combination with the stage 4 opaca endoderm there is a highly significant enhancement in the erythropoietic differentiation of the mesodermal cells (Experiment 4.XV, Section 4.3.3) (p = much less than 0,001).

In the next experiment the effect of the stage 1-2 opaca endoderm

on the erythropoietic potential of the stage 8-9 pellucida mesoderm has been investigated.

Experiment 4.XVII (meso (AP, st 8-9) + endo (AOV, st 1-2))

Stage 8-9 pellucida mesodermal fragments were isolated and combined in culture with stage 1-2 opaca endodermal fragments (Fig. 4.24).

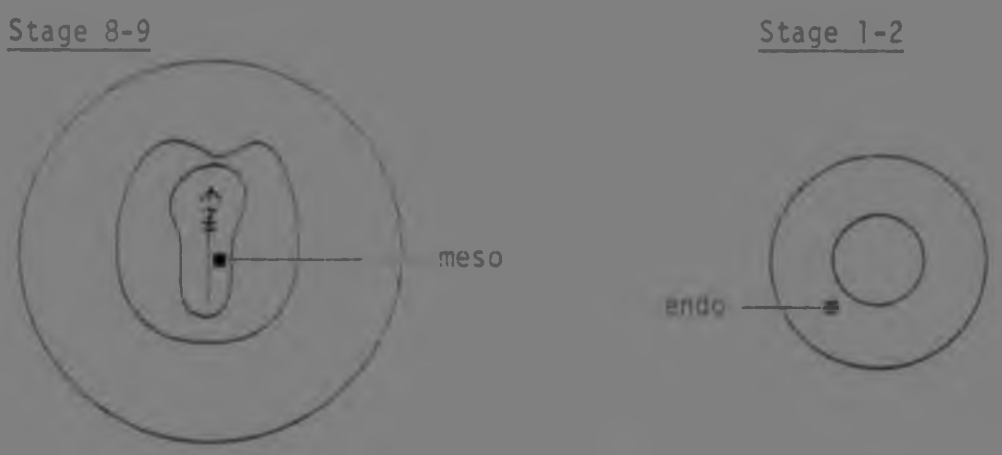


Figure 4.24

Diagram illustrating the areas from where stage 8-9 pellucida mesodermal fragments and stage 1-2 opaca endodermal fragments were isolated for combined culture.

After 72 hours of culture in small drops of medium, the following results were obtained, as tabulated in Table 4.4.

- 62,5% of the explants (10 cases out of 16) stained the blue colour of the counterstain only (i.e., the staining for haemoglobin was negative);
-+ 12,5% of the explants (2 cases out of 16) exhibited traces of pale yellow staining (i.e., uncertain haemoglobin staining);
+ 12,5% of the explants (2 cases out of 16) exhibited pale brown (i.e., pale haemoglobin) staining in a small number of cells;
++ 12,5% of the explants (2 cases out of 16) exhibited more intense haemoglobin staining in a small number of cells.

When the results of this experiment are compared with the results of the experiment in which the stage 8-9 pellucida mesodermal fragments were cultured alone (Experiment 4.II, Section 4.3.1), it is found that

in the presence of the stage 1-2 opaca endoderm there is a highly significant enhancement in the erythropoietic differentiation of the stage 8-9 pellucida mesodermal cells compared to when they are cultured alone (p = less than 0,001). However, this enhancement in the erythropoietic differentiation of the stage 8-9 pellucida mesodermal cells is still significantly lower than that which occurred when the stage 8-9 pellucida mesodermal fragments were cultured in combination with the stage 4 opaca endoderm (Experiment 4.V, Section 4.3.1) (p = less than 0,02).

In the next two experiments the effect of the stage 1-2 opaca environment as a whole (i.e., the stage 1-2 opaca ectoderm-plus-endoderm) on the erythropoietic differentiation of the stage 4- and the stage 8-9 pellucida mesoderm has been investigated.

Experiment 4.XVIII (meso (AP, st 4) plus (ecto + endo (AOV, st 1-2))

Stage 4 pellucida mesodermal fragments were isolated as above and combined on coverslips with stage 1-2 opaca ectoderm-plus-endodermal fragments (Fig. 4.25).



Figure 4.25

Diagram illustrating the areas from where stage 4 pellucida mesodermal fragments and stage 1-2 opaca ectoderm-plus-endodermal fragments were isolated for combined culture.

After 72 hours of culture in small drops of medium, the following results were obtained, as tabulated in Table 4.4.

- 54,5% of the explants (6 cases out of 11) stained the blue colour of the counterstain only (i.e., the staining for haemoglobin was negative);

-+ No explants fell into this category;

+ 27,3% of the explants (3 cases out of 11) exhibited pale brown (i.e., pale haemoglobin) staining in a large number of cells;

++ 18,2% of the explants (2 cases out of 11) exhibited more intense haemoglobin staining in a small number of cells.

Comparing the results of this experiment with those of the experiment in which the stage 4 pellucida mesoderm was cultured in combination with the stage 1-2 opaca endoderm only (Experiment 4.XVI), it is found that the erythropoietic differentiation of the stage 4 pellucida mesoderm in combination with the stage 1-2 opaca endoderm is significantly greater than that of the stage 4 pellucida mesoderm in combination with the stage 1-2 opaca ectoderm-plus-endodermal fragments (p = less than 0,002). Furthermore, when the results of the present experiment are compared with those of the experiment in which the stage 4 pellucida mesoderm was cultured alone (Experiment 4.XII, Section 4.3.3), it is found that the erythropoietic differentiation of the stage 4 pellucida mesoderm cultured alone is likewise significantly greater than that of the stage 4 pellucida mesoderm cultured in combination with the stage 1-2 opaca ectoderm-plus-endoderm (p = less than 0,01). Thus, the stage 1-2 opaca ectoderm appears to exert a negative influence on the erythropoietic differentiation of the stage 4 pellucida mesodermal cells. This situation is analogous to the finding that the stage 4 opaca ectoderm (Section 4.3.2) and the stage 4 pellucida ectoderm (Section 4.3.3) exert an inhibitory effect on mesodermal erythropoiesis.

Coupled with this, it is further found that the present explants tended to spread out and become flattened over the surface of the coverslips during the culturing period. This is identical to the situation which occurred when the stage 4 opaca- and pellucida ectoderm-plus-mesodermal fragments were cultured (Experiments 4.IX and 4.XIII, Sections 4.3.2 and 4.3.3 - see Fig. 4.14). As found in the above two experiments, the flattening of the present explants tended to be more prominent in those cases in which the staining for haemo-

(Text continues on page 216).

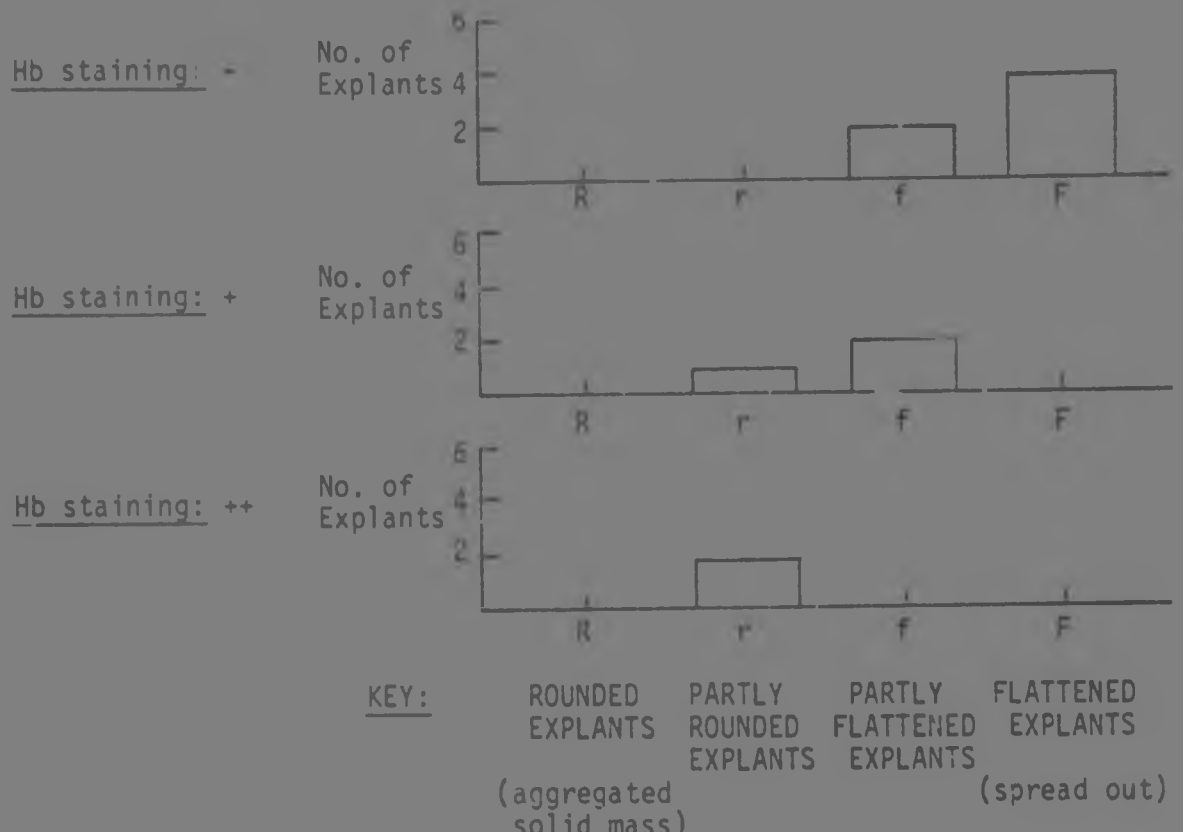


Figure 4.26

Histograms representing the results of Experiment 4.XVIII, in which stage 4 pellucida mesodermal fragments were cultured in combination with stage 1-2 opaca ectoderm-plus-endodermal fragments. The results from each category of haemoglobin staining intensity have been plotted in a separate histogram. Each histogram represents the number of explants in a particular haemoglobin staining category plotted against the degree of tissue spreading (flattening). The results indicate the tendency for the explants in which the staining for haemoglobin is negative (i.e., the explants in category -) to be flattened, whereas the explants in which the staining for haemoglobin is positive (i.e., the explants in categories + and ++) tend to be less flattened. The key representing the increasing degrees of tissue spreading (flattening) is the same as that presented below the histograms of Fig. 4.15.

globin was negative (i.e., the explants in category -), in which cases the explants tended to spread out and form a flattened carpet on the surface of the coverslips (cf. Fig. 4.14, left hand micrograph). In the more 'positive' categories (i.e., the explants in categories + and ++) the tissue tended to be less flattened, although it still displayed the tendency to spread out over the surface of the coverslips (cf. Fig. 4.14, right hand micrograph).

This trend is clearly seen in Fig. 4.26 (page 215), in which the results of the present experiment have been plotted in the form of histograms. As done for Experiments 4.IX and 4.XIII (see Figs. 4.15 and 4.20, respectively), a separate histogram has been plotted for each category of haemoglobin staining intensity. Each histogram represents the number of explants plotted against increasing degrees of tissue flattening.

In the next experiment (Experiment 4.XIX) the stage 1-2 opaca ectoderm-plus-endoderm was cultured in combination with the stage 8-9 pellucida mesoderm.

Experiment 4.XIX (meso (AP, st 8-9) plus)
(ecto + endo (AOV, st 1-2))

Stage 8-9 pellucida mesodermal fragments were isolated as above and combined on coverslips with stage 1-2 opaca ectoderm-plus-endodermal fragments (Fig. 4.27).

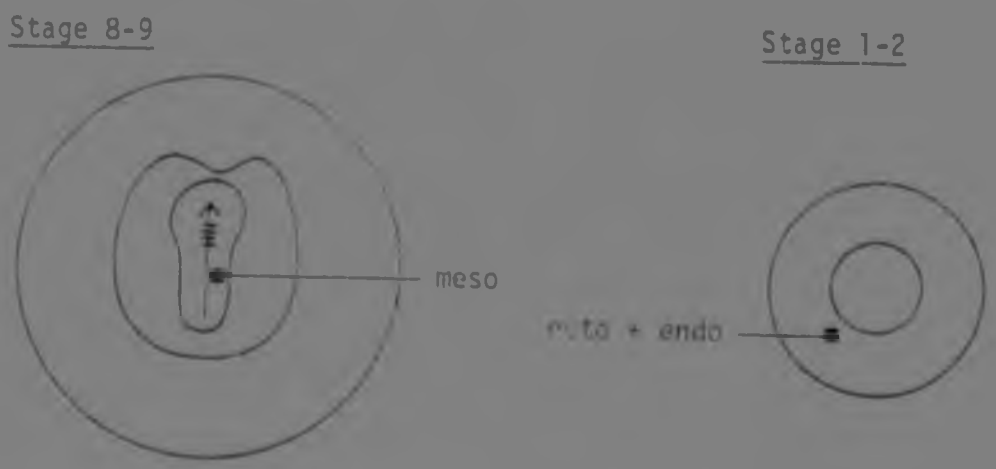


Figure 4.27

Diagram illustrating the areas from where stage 8-9 pellucida mesodermal fragments and stage 1-2 opaca ectoderm-plus-endodermal fragments were isolated for combined culture.

After 72 hours of culture in small drops of medium, the following results were obtained, as tabulated in Table 4.4.

\- 87,5% of the explants (14 cases out of 16) stained the blue colour of the counterstain only (i.e., the staining for haemoglobin was negative);

-+ 6,3% of the explants (1 case out of 16) exhibited traces of pale yellow staining (i.e., uncertain haemoglobin staining);

\+ 6,3% of the explants (1 case out of 16) exhibited pale brown (i.e., pale haemoglobin) staining in a small number of cells.

Comparing the results of this experiment with those of the experiment in which the stage 8-9 pellucida mesoderm was cultured in the presence of the stage 1-2 opaca endoderm only (Experiment 4.XVII), it is found that there is not a significant difference between the erythropoietic differentiation of the stage 8-9 pellucida mesodermal cells when cultured in the presence of the stage 1-2 opaca endoderm or the stage 1-2 opaca ectoderm-plus-endoderm (p = greater than 0,05). Furthermore, when the results of the present experiment are compared with those of the experiment in which the stage 8-9 pellucida mesoderm was cultured alone (Experiment 4.II, Section 4.3.1), it is found that the amount of haemoglobin synthesized by the stage 8-9 pellucida mesoderm when cultured alone is very comparable with that synthesized by the stage 8-9 pellucida mesoderm when cultured in combination with the stage 1-2 opaca ectoderm-plus-endoderm (p = greater than 0,5). Thus, the stage 1-2 opaca environment as a whole (i.e., the stage 1-2 opaca ectoderm-plus-endoderm) does not appear to affect the erythropoietic potential of the stage 8-9 pellucida mesodermal cells.

A further finding in the present experiment, analogous with that described in previous experiments (experiments 4.IX, 4.XIII and 4.XVIII), is that the present explants tended to spread out and become flattened over the surface of the coverslips during the culturing period (cf. Fig. 4.14, left hand micrograph). This situation is clearly shown in Fig. 4.28, in which the results of the present experiment have been plotted in the form of histograms. As done previously, a separate histogram has been plotted for each category of haemoglobin staining intensity. Each histogram represents the number of explants plotted against increasing degrees of tissue flattening.

(Text continues on page 219).

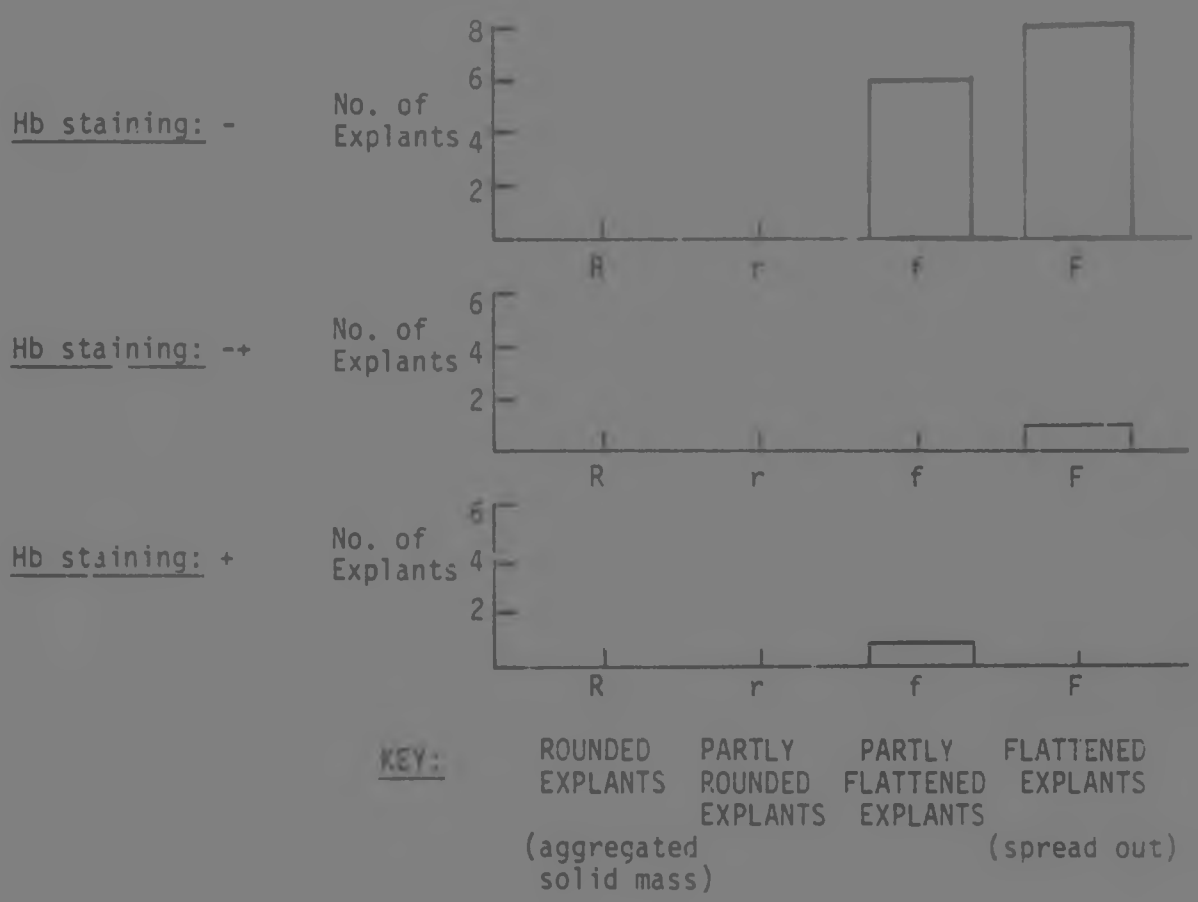


Figure 4.28

Histograms representing the results of Experiment 4.XIX, in which stage 8-9 pellucida mesodermal fragments were combined in culture with stage 1-2 opaca ectoderm-plus-endodermal fragments. The results from each category of haemoglobin staining intensity have been plotted in a separate histogram. Each histogram represents the number of explants in a particular haemoglobin staining category plotted against the degree of tissue spreading (flattening). The results indicate the tendency for the explants to be flattened. The key representing the increasing degrees of tissue spreading (flattening) is the same as that presented below the histograms of Fig. 4.15.

Control cultures

As control cultures for the above experiments, explants composed of the stage 1-2 opaca endoderm (Experiment 4.XX), and explants composed of the stage 1-2 opaca ectoderm-plus-endoderm (Experiment 4.XXI) were cultured alone in small drops of medium (Fig. 4.29).

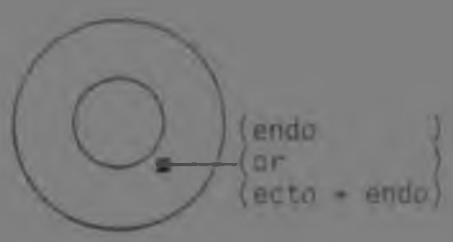


Figure 4.29

Diagram illustrating the area from where stage 1-2 opaca endodermal fragments (Experiment 4.XX) and stage 1-2 opaca ectoderm-plus-endodermal fragments (Experiment 4.XXI) were isolated.

As tabulated in Table 4.4, 100% of these control cultures (10 cases out of 10 in each experiment) exhibited negative staining for haemoglobin. It was once again found that the explants composed of the stage 1-2 opaca ectoderm-plus-endoderm tended to spread out and become flattened over the surface of the coverslips during the culturing period (Experiment 4.XXI). This situation is analogous to that which has been described previously (Experiments 4.IX, 4.XIII, 4.XVIII, and 4.XIX). This spreading and flattening of the explants was not prominent in the explants which were composed of the stage 1-2 opaca endoderm only (Experiment 4.XX).

Routine control cultures were also carried out during the course of all the above experiments to ensure that the culture medium and the o-dianisidine stain were always optimal (Experiment 4.1, page 173). As tabulated in Table 4.4, when explants composed of all three germinal layers were isolated from a normally erythropoietic part of the blastoderm, 100% of the explants (167 cases out of 167) exhibited very intense staining for haemoglobin.

(Text continues on page 221).

Table 4.4 Table summarizing the results of the study to investigate the effects of the stage 1-2 opaca endoderm and ectoderm on mesodermal erythropoiesis (Section 4.3.4).

Experiments	Types of Explants	Total No. of Explants	Categories of Haemoglobin Staining (See Key, Fig. 4.1 page 171). No. of Explants					% of total No.				
			-	-+	+	++	+++	-	-+	+	++	+++
Expt. 4.XVI	meso (AP, st 4) plus endo (AOV, st 1-2)	10	0	0	3	4	3	0,0	0,0	30,0	40,0	30,0
Expt. 4.XVII	meso (AP, st 8-9) plus endo (AOV, st 1-2)	16	10	2	2	2	0	62,5	12,5	12,5	12,5	0,0
Expt. 4.XVIII	meso (AP, st 4) plus ecto + endo (AOV, st 1-2)	11	6	0	3	2	0	54,5	0,0	27,3	18,2	0,0
Expt. 4.XIX	meso (AP, st 8-9) plus ecto + endo (AOV, st 1-2)	16	14	1	1	0	0	87,5	6,3	6,3	0,0	0,0
Expt. 4.XX (Control)	endo (AOV, st 1-2)	10	10	0	0	0	0	100,0	0,0	0,0	0,0	0,0
Expt. 4.XXI (Control)	ecto + endo (AOV, st 1-2)	10	10	0	0	0	0	100,0	0,0	0,0	0,0	0,0
Expt. 4.1 (Routine Control)	ecto + meso + endo (AOV, st 8-9)	167	0	0	0	0	167	0,0	0,0	0,0	0,0	100,0

4 1.4.2 Summary of the investigation of the effects of stage 1-2 opaca ectoderm and endoderm on mesodermal erythropoiesis

The present study has investigated the effects of the stage 1-2 opaca endoderm and ectoderm on the erythropoietic differentiation of the stage 4- and the stage 8-9 pellucida mesoderm.

It was found that the stage 1-2 opaca endoderm, cultured in combination with the stage 4 pellucida mesoderm, did not affect the erythropoietic potential of the stage 4 pellucida mesoderm (Experiment 4.XVI). On the other hand, the stage 1-2 opaca ectoderm-plus-endoderm exerted a negative effect on the erythropoietic differentiation of the stage 4 pellucida mesoderm (Experiment XVIII). This negative effect therefore appeared to be due to the presence of the stage 1-2 opaca ectoderm. (This is analogous to the finding that the stage 4 opaca ectoderm and the stage 4 pellucida ectoderm exert an inhibitory effect on mesodermal erythropoiesis (Sections 4.3.2 and 4.3.3)). Again, this negative effect was coupled with the tendency of the explants to spread out and become flattened over the surface of the coverslips.

When the stage 1-2 opaca endoderm was cultured in combination with the stage 8-9 pellucida mesoderm, there was a significant enhancement in the erythropoietic differentiation of the stage 8-9 pellucida mesodermal cells (Experiment XVII). (However, this enhancement in mesodermal erythropoiesis was still significantly less than that which occurred when the stage 8-9 pellucida mesoderm was cultured in combination with the stage 4 opaca endoderm (Experiment 4.V, Section 4.3.1)). On the other hand, when the stage 8-9 pellucida mesoderm was cultured in combination with the stage 1-2 opaca ectoderm-plus-endodermal fragments, the erythropoietic potential of the stage 8-9 pellucida mesoderm remained low (Experiment 4.XIX). This again was seen to be coupled with the tendency of the explants to spread out and become flattened over the surface of the coverslips.

The results of the present study therefore indicate that the stage 1-2 opaca endoderm may have no effect (Experiment 4.XVI) or a small positive effect (Experiment XVII) on the erythropoietic differentiation of the stage 4- or stage 8-9 pellucida mesodermal cells,

respectively. This indicates that the stage 1-2 opaca endoderm has not yet fully acquired its 'permissive' effects on mesodermal erythropoiesis. On the other hand, the stage 1-2 opaca ectoderm may exert a negative effect on the erythropoietic differentiation of the stage 4 pellucida mesodermal cells (Experiment 4.XVIII). This is coupled with the tendency of these explants to spread out and become flattened.

These findings therefore confirm that the stage 1-2 opaca environment is, due to the inhibitory properties of its ectoderm and the non-permissive properties of its endoderm, not yet a permissive environment for mesodermal erythropoiesis.

4.4 Discussion

Using a coverslip culture technique, the present study evaluates the effects of the component germinal layers of the AP and AOV on the erythropoietic differentiation of the mesodermal cells. It is found that the component germinal layers of the AOV and AP have either stimulatory or inhibitory effects on mesodermal erythropoiesis which influence the extent of mesodermal erythropoiesis observed in these two regions.

In the following discussion, considerations are made of how these influences affect the erythropoietic differentiation of the following types of mesodermal cell populations:

1. Stage 4 opaca mesoderm (i.e., mesoderm which has already entered the AOV and which, during normal development, would subsequently differentiate along erythropoietic lines) (see Section 4.4.1 below);
2. Stage 4 pellucida mesoderm (i.e., mesoderm which is still migrating through the AP and which, during normal development, would subsequently enter into the AOV and differentiate along erythropoietic lines) (see Section 4.4.2 below);
3. Stage 8-9 pellucida mesoderm (i.e., mesoderm from the AP of older blastoderms which, during normal development, would remain in the AP and would not differentiate along erythropoietic lines) (see Section 4.4.3 below).

A further aspect discussed below is the timing of the development of these stimulatory and inhibitory influences (see Section 4.4.4 below).

4.4.1 The effects of the stage 4 opaca endoderm and ectoderm on mesodermal erythropoiesis

In the investigation of the effects of the component germinal layers of the stage 4 AOV on mesodermal erythropoiesis, two key findings have emerged:

1. The endodermal cells of the stage 4 AOV are found to exert a stimulatory effect on mesodermal erythropoiesis, confirming the finding of Wilt (1965a) and Miura and Wilt (1969);
2. The ectodermal cells of the stage 4 AOV are found to exert an inhibitory effect on mesodermal erythropoiesis.

These conclusions are derived from an analysis and comparison of the extent of erythropoiesis in the following explants (Section 4.3.2), the details of which are discussed below:

1. ecto + meso (AOV, st 4) + endo (AOV, st 4) = Expt 4.VIII;
2. ecto + meso (AOV, st 4) = Expt 4.IX;
3. meso (AOV, st 4) = Expt 4.X;
4. meso (AOV, st 4) + endo (AOV, st 4) = Expt 4.XI.

In agreement with the studies of Wilt (1965a) and Miura and Wilt (1969), it is found that the erythropoietic differentiation of the stage 4 pellucida ectoderm-plus-mesodermal explants is significantly enhanced when they are cultured in combination with the stage 4 opaca endoderm (Experiment 4.VIII) compared to when they are cultured alone (Experiment 4.IX). Likewise, it is found that the erythropoietic differentiation of the isolated stage 4 opaca mesoderm is significantly enhanced when it is cultured in combination with the stage 4 opaca endoderm (Experiment 4.XI) compared to when it is cultured alone (Experiment 4.X). Both sets of experiments are therefore in support of a stimulatory effect of the stage 4 opaca endoderm on mesodermal erythropoiesis.

In terms of the effects of the stage 4 opaca ectoderm, however, it is found that the erythropoietic differentiation of the stage 4 opaca mesoderm when cultured in combination with the stage 4 opaca

ectoderm (Experiment 4.IX) is significantly lower than that of the stage 4 opaca mesoderm when cultured alone (Experiment 4.X). Thus, the stage 4 opaca ectoderm is found to exert an inhibitory effect on mesodermal erythropoiesis.

This stimulatory effect of the opaca endoderm and inhibitory effect of the opaca ectoderm is of interest in terms of the spatial relationship of the blood-islands to the endoderm and ectoderm during normal yolk sac vasculogenesis (see Chapter Two). This is clearly demonstrated in Fig. 4.30, in which the blood-islands are seen to be situated in juxtaposition to the opaca endoderm and to be physically separated from the ectoderm by the extracellular spaces. Not only are the blood-islands seen to lie in close approximation to the opaca endoderm, but the SEM sections of the present thesis (Chapter Two), in agreement with the TEM sections of Mato *et al* (1964), show that the cells of the blood islands and the underlying endoderm make physical contact with each other by means of cellular processes extending between them (see Figs. 2.15 and 2.18). No such contact is demonstrated between the blood-islands and the overlying ectoderm.

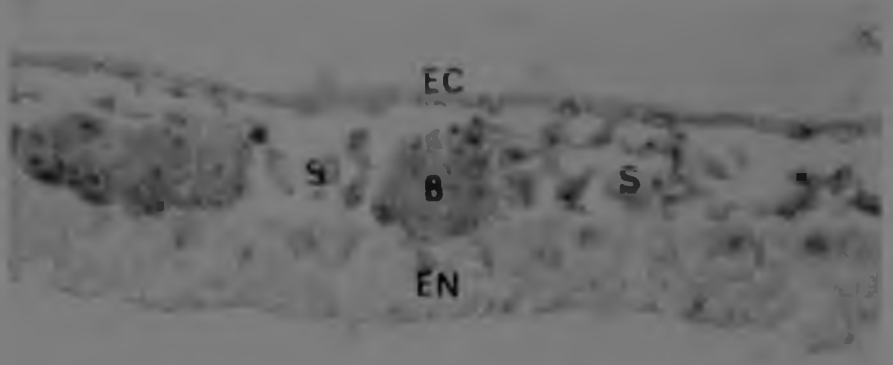


Figure 4.30

Light micrograph of a cross section through the AOV of a stage 6-7 blastoderm to show the spatial relationship of the blood-islands (B) to the endoderm (EN) and ectoderm (EC). The blood-islands are seen to be in juxtaposition to the underlying endoderm and to be separated from the ectoderm by the extracellular spaces. The presumptive coelomic lining cells (S) are situated in the spaces above and between the blood-islands.

B, blood-islands; EC, ectoderm; EN, endoderm;
S, presumptive coelomic lining cells.

x 667

A further finding in the present study is that the inhibitory effect of the opaca ectoderm on mesodermal erythropoiesis is seen to be coupled with the tendency of the ectoderm-plus-mesodermal explants (in the absence of the opaca endoderm) to spread out and become flattened over the surface of the coverslips during the culturing period (Experiment 4.IX). This spreading and flattening of the explants is not prominent when the stage 4 opaca endoderm is recombined with the stage 4 opaca ectoderm-plus-mesoderm (Experiment 4.VIII). Thus, not only is the erythropoietic differentiation of the ectoderm-plus-mesodermal explants greatly enhanced when they are recombined in culture with the opaca endoderm, but the explants in the presence of the opaca endoderm do not exhibit the strong tendency to spread out and become flattened.

Similar observations of the spreading and flattening of the stage 4 opaca ectoderm-plus-mesodermal explants were also made by Miura and Wilt (1969) in their study in which the stage 4 opaca explants were cultured on the surfaces of millipore filters. In agreement with the present findings, these workers likewise reported the tendency of the stage 4 opaca ectoderm-plus-mesodermal explants to move out actively and flatten, and found that this was more pronounced in the explants which were cultured in the absence of the stage 4 opaca endoderm across the filter. Indeed, it was due to this tendency of the explants to flatten and spread that Miura and Wilt (1969) tried out and discarded a number of culturing techniques. Finally, these workers employed a clot of chicken plasma attached to the surfaces of the millipore filters. However, according to these authors, this technique still did not eliminate the tendency of the tissue to flatten, although it may have retarded it (Miura and Wilt, 1969).

It is of relevance to consider this aspect of the spreading and flattening of the explants in terms of the normal cellular patterning and organization which occurs during yolk sac vasculogenesis. As described in the histological study of Chapter Two, and as shown in Fig. 4.30, the opaca endoderm is the surface against which the prospective blood-island cells aggregate together. Thereafter, the differentiating coelomic lining cells spread out as a sheet of cells and form flattened linings over the surfaces of the blood-islands, the

endoderm and the ectoderm (Fig. 4.31). The finding from the present culturing experiments that the stage 4 opaca ectoderm-plus-mesodermal explants exhibit both a low level of mesodermal erythropoiesis and the tendency to spread out and become flattened over the surfaces of the coverslips (Experiment 4.IX) would fit in with the observation (see Fig. 4.31) that the opaca ectoderm is a surface under which the mesodermal cells spread out and form a flattened lining, and is not a surface against which the mesodermal cells aggregate together to form the blood-islands. On the other hand, the finding that the stage 4 opaca ectoderm-plus-mesodermal explants, when recombined with the stage 4 opaca endoderm, exhibit both high levels of mesodermal erythropoiesis and the tendency to form a solid aggregated mass of tissue rather than a flattened sheet of tissue (Experiment 4.VIII) is in line with the observation that the opaca endoderm is the surface against which the blood-island cells aggregate together (and over which the presumptive coelomic lining cells will subsequently spread out and flatten) (Figs. 4.30 and 4.31). It would thus appear that the inhibitory influence exerted by the opaca ectoderm on mesodermal erythropoiesis may well be associated with the tendency of the ectoderm-plus-mesodermal explants, in the absence of the opaca endoderm, to spread out and become flattened (see also Chapter Six).

In this regard, Wilt (1965a) found that when the stage 4 opaca ectoderm-plus-mesodermal fragments were cultured in the absence of the opaca endoderm, blood-islands failed to form (i.e., the prospective blood-island cells did not aggregate together to form the blood-islands). Furthermore, Miura and Wilt (1969) found that only one-third of the explants composed of the stage 4 opaca ectoderm-plus-mesoderm gave rise to blood-islands, but that they were so small that many of them could be seen only after the material has been sectioned and examined under high power. When, on the other hand, the stage 4 opaca ectoderm-plus-mesodermal explants were cultured in the presence of the stage 4 opaca endoderm, not only was the spreading of the tissue less evident, but in addition, very obvious blood-islands were formed (Wilt, 1965a; Miura and Wilt, 1969; and the present observations). These findings suggest that in the absence of the opaca endoderm, the spreading and flattening of the ectoderm-plus-mesodermal explants may prevent the formation of the blood-islands, e.g., it may physically prevent the

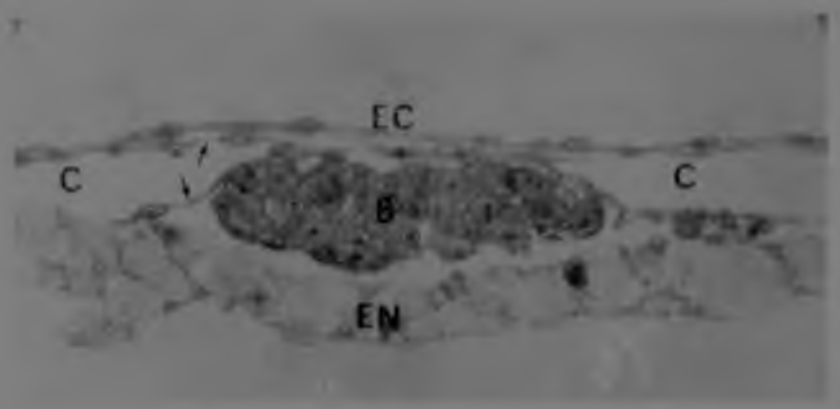


Figure 4.31

Light micrograph of a cross section through the AOV of a stage 9 chick blastoderm. The coelomic lining cells (arrows) are seen to be in the form of flattened cellular linings which underlie the ectoderm (EC) and cover over the surfaces of the blood-island (B) and the endoderm (EN).

B, blood-island; C, coelom; EC, ectoderm; EN, endoderm; arrows, coelomic lining cells.

x 417

clumping together of the prospective blood-island cells to form the blood-islands themselves. This may be associated with the nature of the onaca ectoderm, being a surface under which the mesodermal cells spread rather than aggregate together (see Fig. 4.31).

That the local epithelium may indeed exert an effect on the extent of mesodermal cell spreading has been demonstrated in other tissues in the studies of Fisher and Solursh (1979a,b). By carrying out transplantation experiments, Fisher and Solursh (1979a) showed, for example, that the normally non-migratory limb mesenchyme is capable of spreading like sclerotome when placed in the somite region. Conversely, somites placed in the limb lose their epithelial organization and become organized into the typical limb-like pattern of dispersed cells surrounding a central condensed core of cells. In these studies it was found that the mesenchymes showed varying degrees of cell dispersion in accordance with the cell distance from the associated ectoderm. This type of finding is in line

with the present observations of the degree of cell spreading in the AOV, i.e., the yolk sac ectoderm is seen to be the surface under which the yolk sac mesodermal cells spread, whereas the yolk sac endoderm is the surface against which the blood-island cells clump (Figs. 4.30, 4.31).

Fisher and Solursh (1979b) extended their study to an *in vitro* investigation of the effects of a variety of artificial and natural substrata on the spreading behaviour of these migratory and non-migratory mesenchymes. This study confirmed the view that the spreading of the mesenchymes is largely determined by the properties of the available substratum. In particular, these authors found that isolated basal laminae provided a substratum which supported extensive spreading of the mesenchymal cells. This observation complements the earlier TEM observation of Trelstad *et al* (1967) and Hay (1968) who suggested that the ectodermal basement membrane of the early chick blastoderm serves as a substratum for the spreading of the mesenchymal cells.

In a subsequent chapter of this thesis the effect of physical flattening on mesodermal erythropoiesis has been investigated further (Chapter Six). In this study experiments have been carried out in which the tissue fragments have been flattened by surface tension forces or by the application of mechanical pressure. It is found that the erythropoietic differentiation of the opaca explants may indeed be suppressed if the explants are physically flattened (see Chapter Six).

4.4.2 The effects of the stage 4 pellucida endoderm and ectoderm on mesodermal erythropoiesis

The mesodermal layer of the stage 4 AP comprises a migratory population of cells, most of which will subsequently enter into the AOV and contribute towards the formation of the yolk sac vascular system (see Chapter Two). The question was therefore posed as to why, during normal development, the potentially erythropoietic stage 4 pellucida mesodermal cells do not differentiate erythropoietically in the AP, but continue to migrate centrifugally into the AOV, where they then undergo their erythropoietic differentiation. Using the coverslip culture technique to investigate the effects of the component germinal layers of the stage 4 AP on the erythropoietic differentiation of these cells,

it has been found that:

1. The stage 4 pellucida ectoderm (like the stage 4 opaca ectoderm) exerts an inhibitory effect on mesodermal erythropoiesis;
2. The stage 4 pellucida endoderm (in contrast to the stage 4 opaca endoderm) does not exert a stimulatory effect on mesodermal erythropoiesis;
3. Thus, the environment of the stage 4 AP as a whole (in contrast to that of the AOV) is unfavourable for mesodermal erythropoiesis.

These conclusions are derived from the comparisons of the extent of erythropoiesis in the following tissue combinations (Section 4.3.3), the details of which are discussed below:

1. meso (AP, st 4) = Expt 4.XII;
2. ecto + meso (AP, st 4) = Expt 4.XIII;
3. ecto + meso + endo (AP, st 4) = Expt 4.XIV;
4. meso (AP, st 4) + endo (AOV, st 4) = Expt 4.XV.

An inhibitory effect of the stage 4 pellucida ectoderm on mesodermal erythropoiesis was demonstrated in the observation that the erythropoietic differentiation of the isolated stage 4 pellucida mesoderm cultured alone (Experiment 4.XII) is significantly greater than that of the stage 4 pellucida mesoderm cultured in combination with the stage 4 pellucida ectoderm (Experiment 4.XIII). Thus, the stage 4 pellucida ectoderm, like the stage 4 opaca ectoderm, is found to exert an inhibitory effect on mesodermal erythropoiesis. Again, this inhibitory effect of the ectoderm is seen to be coupled with the tendency of the ectoderm-plus-mesodermal explants to spread out and become flattened over the surface of the coverslips during the culturing period. However, in a few cases these pellucida ectoderm-plus-mesodermal explants, rather than flattening, were found to have formed epithelial vesicles surrounding a central mass of cells (Experiment 4.XIII). Furthermore, the erythropoietic differentiation of these latter explants was likewise low or absent. It would thus appear that other factors besides the spreading and flattening of the explants must also be involved in the inhibitory effect of the ectoderm on mesodermal erythropoiesis.

In terms of the effects of the stage 4 pellucida endoderm on mesodermal erythropoiesis, it is found that the erythropoietic differentiation of the stage 4 pellucida ectoderm-plus-mesodermal explants (Experi-

ment 4.XIII) is not significantly different from that of the stage 4 pellucida ectoderm-plus-mesoderm when cultured in combination with the stage 4 pellucida endoderm (Experiment 4.XIV). This indicates that the negative influence exerted by the stage 4 pellucida ectoderm on mesodermal erythropoiesis is not overcome by the presence of the stage 4 pellucida endoderm. Coupled with this, the stage 4 pellucida explants composed of all three germinal layers still exhibit the tendency to spread out and become flattened over the surface of the coverslips. Thus, the environment of the stage 4 AP as a whole is found to be unfavourable for mesodermal erythropoiesis. (This situation is in contrast to the observations made in the AOV, in which the stimulatory effects of the stage 4 opaca endoderm are seen to overcome the inhibitory effects of the stage 4 opaca ectoderm so that the environment of the AOV as a whole is found to be favourable for mesodermal erythropoiesis - see Sections 4.3.2 and 4.4.1).

A significant finding in the present study is that when the stage 4 pellucida mesoderm is combined in culture with the stage 4 opaca endoderm (Experiment 4.XV), its erythropoietic differentiation is dramatically enhanced to a level comparable with that of the stage 4 opaca mesoderm in combination with the stage 4 opaca endoderm (Experiment 4.XI). This indicates that the stage 4 opaca endoderm is capable of stimulating the potentially erythropoietic stage 4 pellucida mesodermal cells to realize their full erythropoietic differentiation.

These observations fit in well with the temporal and spatial patterns of morphogenesis which are seen to occur during normal embryogenesis (see Chapter Two). The finding that the stage 4 pellucida ectoderm and endoderm comprise an environment which is unfavourable for mesodermal erythropoiesis would explain why, during normal development, the potentially erythropoietic mesodermal cells which are migrating through the AP are not stimulated to differentiate erythropoietically in the AP. On the other hand, the finding that the stage 4 opaca endoderm stimulates the potentially erythropoietic mesodermal cells to realize their full erythropoietic potentiality is in line with the observation that the stage 4 pellucida mesodermal cells will eventually enter into the AOV itself where they will come into contact with the opaca endoderm, and some of them will then aggregate together against the opaca endoderm to form the blood-islands. It is thus seen

that the ectoderm and endoderm of the AF and AOV exert temporally and spatially appropriate morphogenetic influences on the mesodermal cells as they pass through these two parts of the blastoderm.

Of direct relevance to the present investigation are the studies of previous authors on the timing of the initial signs of erythropoiesis in these mesodermal cells. As described in Chapter Two (Section 2.4.4), studies by Chan *et al* (1974) using ^{3}H-labelled cDNA as a probe to detect and quantify chick globin mRNA have shown that whereas only minimal quantities of globin mRNA are present in the potentially erythropoietic mesodermal cells of the stage 4 blastoderm (DPS stage; 18 hours incubation), most of the globin mRNA has already been synthesized by these cells by stage 6 (the head-fold stage; 24 hours incubation), the time at which the blood-island cells are already seen to aggregate together in the AOV. These findings complement the earlier observations of Wilt (1965b), Hagopian *et al* (1972), Chan and Ingram (1973), and Ingram *et al* (1974) that the treatment of stage 4 chick blastoderms with actinomycin D (an inhibitor of RNA synthesis) or 5-bromodeoxyuridine (a thymidine analogue which blocks cell differentiation) completely blocks the subsequent synthesis of haemoglobin which is normally detectable by the 7 somite stage (stage 9; 34 hours incubation), whereas the application of these agents after the head-fold stage (stage 6; 24 hours incubation) permits the subsequent synthesis of haemoglobin to occur.

These observations therefore indicate that the synthesis of most of the globin mRNA occurs during the period between the DPS stage (stage 4; 18 hours incubation) and the head-fold stage (stage 6; 24 hours incubation). This is the period during which the mesodermal cells are migrating through the AP into the AOV, and are clumping together against the yolk sac endoderm (see Chapter Two). It would be of interest to determine whether, during normal development, the synthesis of globin mRNA occurs prior to, or as a consequence of, the entry of the mesodermal cells into the AOV and their contact with the opaca endoderm.

It has further been shown by Chan (1976) from an electrophoretic investigation of the initial time of the translation of this globin mRNA that globin polypeptides are already being synthesized at stage 6 (the head-fold stage; 24 hours incubation). This corresponds to the period during normal development in which the blood-island cells have already started to clump together against the yolk sac endoderm. It would be

of interest to determine whether this synthesis of globin is a cause or a consequence of the clumping together of these cells. Further, in view of the observed correlation recorded in the present work between the tendency of some of the explants to become flattened and the depression in their erythropoietic differentiation, it would be of relevance to determine whether, or to what degree, the mesodermal cells in these present explants synthesize globin mRNA and globin polypeptides. An answer to these questions may be obtained by treating the explants with fluorescein-labelled anti-globin antibody (for globin detection) or ^{3}H-labelled cDNA complementary to globin mRNA (for globin mRNA detection) and determining, in histological sections, whether a correlation exists between the tendency of the mesodermal cells to clump together, and the synthesis by these cells of globin mRNA or globin polypeptides.

4.4.3 The pluripotency of the stage 8-9 pellucida mesodermal cells

The finding from the above investigation that the environment of the AP is unfavourable for mesodermal erythropoiesis whereas the opaca endoderm is stimulatory for mesodermal erythropoiesis, is further confirmed in the study in which the erythropoietic potential of the stage 8-9 pellucida mesoderm has been investigated (Section 4.3.1; see also Chapter Three).

During normal embryogenesis the yolk sac mesoderm in the AP of the stage 8-9 blastoderm does not migrate into the AOV, but remains in the AP where it subsequently differentiates into the endothelial linings and coelomic linings of the AP (see Chapters Two and Three). As indicated previously, and in agreement with the fate maps of Settle (1954), the differentiation of these mesodermal cells tends to occur along non-erythropoietic lines (Chapters Two and Three). However, the present study demonstrates that some of these stage 8-9 pellucida mesodermal cells are still pluripotent, and that they can still differentiate erythropoietically if they are cultured in combination with the opaca endoderm. These findings come from observations and comparisons of the extent of erythropoiesis in the following tissue combinations (Section 4.3.1), the details of which are discussed below:

1. meso (AP, st 8-9) = Expt 4.II;
2. ecto + meso (AP, st 8-9) = Expt 4.III;

3. ecto + meso + endo (AP, st 8-9) = Expt 4.IV;
4. meso (AP, st 8-9) + endo (AOV, st 4) = Expt 4.V;
5. ecto + meso (AP, st 8-9) + endo (AOV, st 4) = Expt 4.VI.

The normally non-erythropoietic nature of the stage 8-9 pellucida mesoderm is demonstrated in the observation that the stage 8-9 pellucida mesoderm when cultured in its normal combination with the stage 8-9 pellucida ectoderm and endoderm (Experiment 4.IV), or in combination with the stage 8-9 pellucida ectoderm only (Experiment 4.III), does not exhibit an erythropoietic differentiation. This is in agreement with the observations from the transplantation experiments of the previous chapter in which the stage 8 pellucida mesodermal fragments transplanted back into the AP of stage 8 host blastoderms, do not differentiate along erythropoietic lines (Chapter Three, Experiment 3.IV). On the other hand, it is found that when the stage 8-9 pellucida mesoderm is isolated from the pellucida ectoderm and endoderm and is cultured alone (Experiment 4.II), there is suggestive evidence that some of its cells may exhibit some potential for an erythropoietic differentiation. This comes from the observation that approximately 40% of the stage 8-9 pellucida mesodermal explants, instead of staining the blue colour of the counterstain only, stained a pale pink colour, and many of their cells exhibited the spindle-shape or boat-shape morphology typical of the free-floating erythrocytes of normal erythropoietic cultures. However, the confirmation of whether these boat-shaped cells are indeed exhibiting an erythropoietic differentiation would have to come from the use of a more sensitive haemoglobin-detection technique than the o-dianisidine stain. Possible methods which could be used would be the use of fluorescein-labelled anti-globin antibody (for globin detection), or ^{3}H-labelled cDNA complementary to globin mRNA (for globin mRNA detection). If these pink boat-shaped cells are indeed found to be exhibiting some potential for an erythropoietic differentiation, this would provide further support for the contention that the environment of the AP is unfavourable for mesodermal erythropoiesis.

The demonstration of the pluripotency of the stage 8-9 pellucida mesodermal cells is shown by the finding that when the stage 8-9 pellucida mesoderm, or the stage 8-9 pellucida ectoderm-plus-mesoderm, is cultured in combination with the stage 4 opaca endoderm, there is a significant enhancement in the erythropoietic differentiation of the

mesodermal cells (Experiments 4.V and 4.VI). This observation supports the finding from the mesodermal transplantation experiments of the previous chapter that some of the normally non-erythropoietic stage 8 pellucida mesodermal cells may differentiate erythropoietically if they are heterotopically transplanted into the AOV of stage 4-5 host blastoderms (Chapter Three, Experiment 3.I). These findings therefore confirm the view that some of the stage 8-9 pellucida mesodermal cells are still pluripotent, and that the environment of the stage 4 AOV, or indeed, the stage 4 opaca endoderm itself, is able to change the pathways of differentiation of these cells, causing them to differentiate along erythropoietic lines. However, during normal development, these stage 8-9 pellucida mesodermal cells would remain in the AP which is unfavourable for mesodermal erythropoiesis, and would therefore not be stimulated to differentiate erythropoietically.

It is pertinent to view these findings in terms of the observations described previously of the so-called 'intermediate zone' in the border region of the AP with the AOV, in which the pellucida mesodermal cells are found to exhibit some potential for an erythropoietic differentiation (see Chapter Two). This phenomenon is logically explained from the above observations of:

(1) the pluripotency of the mesodermal cells;
(2) the inhibitory effects of the AP on mesodermal erythropoiesis; and
(3) the stimulatory effects of the AOV (or indeed, the opaca endoderm) on mesodermal erythropoiesis.

It would appear that the mesodermal cells in this 'intermediate zone' are subjected to both the inhibitory effects of the AP and the stimulatory effects of the opaca endoderm. This would explain why these cells tend to give rise to blood vessels containing only small numbers of blood cells (see Chapter Two).

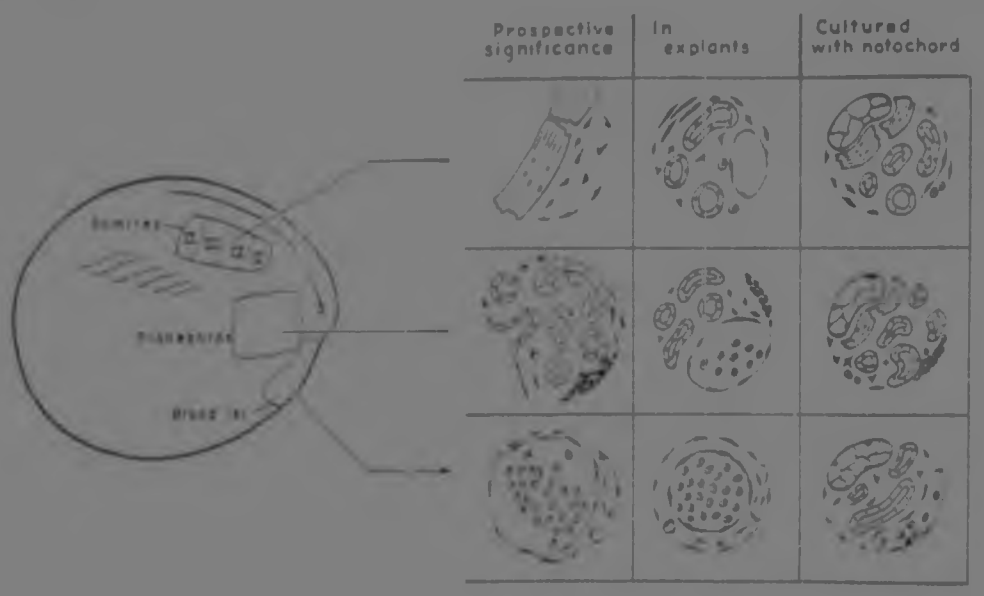
Further insight into the roles of the surrounding tissues in moulding the graded morphogenetic patterning of the mesodermal cells comes from the studies of Yamada (1937, 1940), Muchmore (1951) and Finnegan (1953) on the mesodermal mantle of the early urodele embryo (see also the review by Holtfreter and Hamburger, 1955). Yamada (1940) and Muchmore (1951) studied the developmental potentials of the various

presumptive regions of the amphibian mesodermal mantle, which are normally organized in the 'descending' series: somites, pronephros, and blood-islands (Fig. 4.32). When explants of these presumptive regions were isolated in ectodermal vesicles, they were found to exhibit a grade of differentiation 'below' their prospective fates (Fig. 4.32). Thus, the presumptive somite mesoderm produced pronephros, blood cells and blood vessels instead of skeletal muscle; the presumptive pronephros produced blood cells and blood vessels in addition to pronephros, the latter sometimes being poorly developed (Fig. 4.32). (In the above situation, the presumptive blood-island mesoderm produced blood cells and blood vessels only, in accordance with its prospective significance (Fig. 4.32)). On the other hand, it was found that if the presumptive notochord was added to the explants, their morphogenetic potential was 'increased' (Yamada, 1940; Muchmore, 1951) (Fig. 4.32). Thus, the presumptive pronephros produced skeletal muscle in addition to pronephric tubules, with blood cell production being much decreased; the presumptive blood-island tissue produced nephric tubules and in some cases even skeletal muscle, with blood cell production likewise being decreased (Fig. 4.32).

Using this experimental data, Yamada (1940, 1947) and Muchmore (1951) have hypothesized possible mechanisms to explain the determination of the pattern of differentiation of the mesodermal mantle.

According to the model of Yamada (1940), there exists in the mesodermal mantle of the developing amphibian embryo a dorso-ventral gradient of morphogenetic potential which is dependent upon some 'factor' originating in the notochord and spreading ventrally through the mesodermal mantle. Thus, the dorsal mesoderm contains the greatest and the ventral mesoderm the least amount of this 'chorda factor'. In terms of this model, the specific differentiation of the mesoderm in any given region depends upon the value of this potential with respect to certain threshold values: muscle differentiation corresponds to the highest value, tubule differentiation to a lower value, and blood cell differentiation to the lowest value of this potential. (To explain certain observations in development which do not agree with this evenly dispersed gradient of polarity across the mesodermal mantle, Yamada (1947) subsequently postulated an additional cephalo-caudal gradient of potential, proposing that ary given region of the embryo can be charac-

terized by definite values of both dorso-ventral and cephalo-caudal potentials (see Yamada (1950) for a review)).



(After Yamada, 1940 from Holtfreter and Hamburger, 1955).

Figure 4.32

Schematic representation of the pathways of differentiation of the prospective somite, pronephros, and blood-island regions of the early urodele neurula under the following conditions:
First vertical row : normal fate;
Second vertical row: differentiation when isolated in ectodermal vesicle;
Third vertical row : differentiation when combined with notochord, in ectodermal vesicle.

On the other hand, Muchmore (1951) envisaged the organization of the mesodermal mantle in terms of several overlapping morphogenetic fields, characterized by qualitative rather than quantitative differences. In terms of this model, the final differentiation of this system depends upon the predominating interactions of these fields. For example, the presumptive somite region may lie within at least three fields, i.e., chorda, muscle and pronephros, with the muscle field being predominant in normal development.

It is tempting to apply these models of Yamada (1940, 1947) and Muchmore (1951) to explain the graded type of morphogenesis of the

yolk sac blood system in the AP and AOV of the chick blastoderm. Although in the present study attention was not focused directly on the notochord, somite or kidney regions (i.e., the components of the embryonic axis itself), it was nevertheless clearly shown that the pellucida environment is unfavourable for mesodermal erythropoiesis whereas the opaca environment is favourable for mesodermal erythropoiesis. This fits in with the models of Yamada and Muchmore, in that the unfavourable pellucida environment is close to the embryonic axis (hence the notochord) whereas the favourable opaca environment is distantly removed from it (see observations in Chapters Two, Three, and the present chapter). It would be interesting to repeat the explantation studies of Yamada (1940) and Muchmore (1951) on the presumptive somite, kidney and blood-island regions of the chick blastoderm to determine whether a similar hierarchy of developmental properties or 'fields' exists in the chick. In this regard, it has already been shown that hensens node or the presumptive notochord transplanted into a more lateral position in the chick blastoderm can induce a second embryonic axis (Waddington and Schmidt, 1933; Mulherkar, 1958; Gallera, 1964, 1966; see also the review by Bellairs, 1971). Furthermore, it has been found that this inducing capacity falls off not only along the longitudinal axis of the primitive streak, but also laterally in the AP (Mulherkar, 1958). It may well be that the high point of axis induction inhibits blood cell formation.

On the other hand, the present observations and those of Wilt (1965a) and Miura and Wilt (1969) that the opaca endoderm positively stimulates mesodermal erythropoiesis would lead one to further propose some 'morphogenetic field' or influence emanating from the opaca endoderm which strongly favours blood cell formation. This type of prediction is in line with observations made in the amphibian embryo by Finnegan (1953), who found that when the presumptive blood-island tissue of the neurula or tail-bud embryo was transplanted into a more dorsal region of a host embryo of similar age, it failed to differentiate erythropoietically (as expected) unless its underlying endoderm was included in the transplant. In the presence of its endoderm, complete erythropoiesis occurred even though the tissue had been transplanted into the dorsal region of the embryo. As concluded by Finnegan, ". . . erythropoiesis is furthered by the proximity of the

endodermal elements which assist in establishing and maintaining an erythropoietic environment." (Finnegan, 1953 page 391). The present observations that the presence of the stage 4 opaca endoderm results in a significant enhancement in the erythropoietic differentiation of the stage 4 pellucida mesoderm (Experiment 4.XV, Section 4.3.3) and, furthermore, causes some of the normally non-erythropoietic stage 8-9 pellucida mesodermal cells to differentiate erythropoietically (Experiments 4.V and 4.VI, Section 4.3.1) are in support of a strong stimulatory effect of the opaca endoderm on mesodermal erythropoiesis.

Thus, the proximo-distal gradation in the developmental properties of the yolk sac vascular system, as seen in the extent of the development of the blood cells in the AP and AOV, can well be seen to be a consequence of at least two types of influence, an inhibitory one emanating from the AP and a stimulatory one emanating from the opaca endoderm.

An important difference noted between the explantation studies of Yamada (1940) and Muchmore (1951) on the amphibian embryo, and the present explantation study on the chick blastoderm, is that whereas in the present study the chick ectoderm from both the AP and AOV is found to have an inhibitory effect on mesodermal erythropoiesis, Yamada (1940) and Muchmore (1951) did not find an inhibitory effect of the amphibian ectoderm on mesodermal erythropoiesis (see Fig. 4.32). Indeed, whereas in the present study the erythropoietic differentiation of the explants composed of the ectoderm-plus-mesoderm was found to be significantly depressed (see Sections 4.3.2 and 4.3.3), in the experiments of Yamada (1940) and Muchmore (1951) the mesoderm cultured with the ectoderm tended to differentiate in the direction of blood cell formation (Fig. 4.32).

It is tempting to explain this difference in terms of the present observation that the inhibitory effect of the ectoderm on mesodermal erythropoiesis tends to be coupled with the tendency of the explants to spread out and become flattened in culture, whereas in the experiments of Yamada (1940) and Muchmore (1951) the explants did not become flattened, but were in the form of the mesoderm within an ectodermal vesicle. However, it should be noted that in the present study there were also a number of cases in which the pellucida ectoderm-plus-

mesodermal explants likewise took on the appearance of an epithelial vesicle surrounding a central mass of tissue (Experiment 4.III, Section 4.3.1; Experiment 4.XIII, Section 4.3.3). Furthermore, in these latter cases the erythropoietic differentiation of the explants was likewise low or absent. Thus, care is in order when deciding whether the inhibitory effect of the ectoderm on mesodermal erythropoiesis is due to this flattening and spreading of the explants, or whether other factors besides the flattening of the explants are responsible for the inhibitory effect of the ectoderm on mesodermal erythropoiesis (see also Chapter Six).

In subsequent chapters of this thesis further aspects of the question concerning the role of environmental influences in moulding the morphogenetic patterning of the mesodermal cells are discussed (see Chapters Five, Six and Eight).

4.4.4 The effect of the stage 1-2 opaca endoderm and ectoderm on mesodermal erythropoiesis

Whereas the stage 4 opaca endoderm is consistently shown (in both the present work and that of Wilt (1965a) and Miura and Wilt (1969)) to exert a stimulatory effect on mesodermal erythropoiesis, the present study demonstrates that the very early (stage 1-2) opaca environment is still unfavourable for mesodermal erythropoiesis.

This conclusion comes from observations and comparisons of the extent of erythropoiesis in the following tissue combinations (Section 4.3.4), the details of which are discussed below:

1. meso (AP, st 4) + endo (AOV, st 1-2) = Expt 4.XVI;
2. meso (AP, st 8-9) + endo (AOV, st 1-2) = Expt 4.XVII;
3. meso (AP, st 4) + ecto + endo (AOV, st 1-2) = Expt 4.XVIII;
4. meso (AP, st 8-9) + ecto + endo (AOV, st 1-2) = Expt 4.XIX.

It is found that whereas the stage 1-2 opaca endoderm has no effect on the extent of erythropoiesis of the stage 4 pellucida mesodermal cells (Experiment 4.XVI), it exerts a small positive effect on the erythropoietic differentiation of the normally non-erythropoietic stage 8-9 pellucida mesodermal cells (Experiment 4.XVII). Thus, the stage 1-2 opaca endoderm is found to have no effect, or a small positive effect, on mesodermal erythropoiesis.

That the stage 1-2 opaca ectoderm is capable of exerting a negative effect on mesodermal erythropoiesis is demonstrated in the observation that the erythropoietic differentiation of the stage 4 pellucida mesoderm is significantly depressed when it is cultured in combination with the stage 1-2 opaca ectoderm-plus-endoderm (Experiment 4.XVIII). On the other hand, the erythropoietic differentiation of the stage 8-9 pellucida mesoderm in combination with the stage 1-2 opaca ectoderm-plus-endoderm remains low, as normal (Experiment 4.XIX). This indicates that at these very early stages (i.e., stages 1-2) the opaca ectoderm is already capable of exerting its negative effect on mesodermal erythropoiesis. Again, this inhibitory effect is found to be coupled with the tendency of the explants to spread out and become flattened over the surface of the coverslips during the culturing period.

These findings therefore indicate that the stage 1-2 opaca environment, in view of the inhibitory properties of its ectoderm and the non-permissive properties of its endoderm, is not yet a stimulatory environment for mesodermal erythropoiesis.

In terms of normal embryogenesis, the stage 1-2 blastoderm (pre-streak to initial streak; up to 6-7 hours incubation) is the developmental stage at which the presumptive mesodermal cells are just starting to invaginate from the epiblast through the primitive streak. This is at least ten to twelve hours before these mesodermal cells would eventually enter into the AOV (see Chapter Two). Since the opaca endoderm at these very early stages is capable of exerting, at most, only a small positive effect on mesodermal erythropoiesis, it would appear that the stimulatory influence of the opaca endoderm on mesodermal erythropoiesis must develop, or be acquired, during the ten to twelve hour period between the initial streak stage (stage 1-2) and the DPS stage (stage 4). Bennett (1973), using the enzyme cysteine lyase as a marker of endodermal cell differentiation, reported that the differentiation of the opaca endoderm is stimulated by the yolk sac mesoderm and is temporally coupled with the migration of the mesoderm into the AOV. It is therefore tempting to speculate that the stimulatory effect of the opaca mesoderm on endodermal differentiation may well be responsible for the endoderm, in turn, becoming stimulatory to the erythropoietic differentiation of the mesoderm itself.

In terms of the present results, however, this does not appear to be the case since it is found that the stage 1-2 opaca endoderm cultured in combination with the stage 4 pellucida mesoderm does not enhance the erythropoietic differentiation of the stage 4 pellucida mesoderm (Experiment 4.XVI). This would indicate that the interaction between the stage 4 pellucida mesoderm and the stage 1-2 opaca endoderm does not result in the s 1-2 opa endoderm becoming stimulatory for mesodermal ery s. However, caution is in order when interpreting these fi ngs because it may well be that the stage 1-2 opaca endoderm has not yet acquired the competence to react to the stage 4 pellucida mesoderm. During normal embryogenesis the stage 1-2 opaca endoderm would only come into contact with this mesoderm when it is ten to twelve hours older, a time period during which it could have well acquired the competence to interact with the mesoderm.

Further research is therefore required to determine nd how the opaca endoderm becomes stimulatory for mesodermal erythr poiesis, and indeed, what the molecular basis of this stimulatory effect is.

CHAPTER FIVE

THE ROLE OF COMPONENTS OF THE EXTRACELLULAR MATRIX, HYALURONATE AND COLLAGEN, IN YOLK SAC VASCULOGENESIS AS INVESTIGATED BY THE COVERSLIP CULTURE OF BLASTODERM FRAGMENTS IN SMALL DROPS OF MEDIUM

5.1 Introduction

In the previous chapter the inhibitory influence of the ectoderm and the stimulatory influence of the stage 4 opaca endoderm on the erythropoietic differentiation of the mesodermal cells have been demonstrated. In the general area of cell-cell interactions, the interactions between cells and the extracellular matrix are also believed to play an important role during morphogenesis and cyto-differentiation (see Manasek, 1975; Lash and Burger, 1977; Adamson 1981).

The cells of the blastoderm are in intimate association with the contents of the extracellular matrix, which comprise the immediate environment within which the cells perform their various functions. The components of the extracellular matrix include macromolecules, water and ions (see Manasek, 1975). The major macromolecular components are the glycosaminoglycans, glycoproteins and the different types of collagens (see Section 5.3.2 for a discussion of the different collagen types). The embryonic extracellular environment is dynamic, there being continual changes in the composition and distribution of its macromolecular components as the differentiation of the cells themselves proceeds (see e.g. Von der Mark *et al*, 1976a,b; Dessau *et al*, 1980; Thesleff *et al*, 1979, 1981). This is highlighted, for example, in the work of von der Mark and coworkers who, using immunofluorescence techniques, demonstrated a change in the distribution and concentration of fibronectin and Type I collagen during the different developmental stages of the developing chick limb (Von der Mark *et al*, 1976a,b; Dessau *et al*, 1980). Thus, during the early stages of limb bud formation, prior to the condensation of the core mesenchyme and the formation of the cartilage blastema, fibronectin and Type I collagen are found to be evenly distributed throughout the intercellular spaces of the limb mesenchyme. With the onset of the condensation process, fibronectin and Type I collagen are found to increase in the cartilage blastema

compared to the surrounding loose mesenchyme, reaching a maximum density at the time of cartilage differentiation. This latter process is marked by the appearance of Type II collagen in the cartilage blastema. As cartilage differentiation progresses, fibronectin disappears and Type I collagen is gradually replaced by Type II collagen (see Dessau *et al*, 1980). It is thus found that the transient appearance of fibronectin and Type I collagen is temporally coupled with the morpnogenesis and differentiation of the chick limb bud mesenchymal cells.

Analogous observations of the synthesis and removal of particular extracellular matrix components at particular stages of development have also been demonstrated in a number of other developmental systems (e.g. Thesleff *et al*, 1979, 1981; see also review by Toole *et al*, 1977). This dymanic nature of the extracellular matrix suggests that the changing components of the extracellular matrix may be involved in the cell-matrix and/or cell-cell interactions involved in the successive stages of morphogenesis and cytodifferentiation. It may well be that the control of the accumulation and removal of these extracellular proteins may be one mechanism by which the differentiation of the cells may be regulated (see Toole *et al*, 1977). However, care should be exercised in the interpretation of cause and effect.

In the present chapter the coverslip culture technique has been used to investigate the effects of the removal of the extracellular matrix components hyaluronate and collagen[1] on mesodermal erythropoiesis. In the first section attention has been focused on the effects of the removal of hyaluronate (by hyaluronidase treatment) on mesodermal erythropoiesis (Section 5.3.1). In the next section attention has been focused on the effects of the removal of collagen (by collagenase treatment) on mesodermal erythropoiesis (Section 5.3.2).

5.2 Materials and Methods

0,5mm x 0,5mm explants of the chick blastoderm were cultured in small drops of medium on the surface of coverslips below cavity slides, as des-

---

1. The collagen types present in both the AP and AOV of the early chick blastoderm (e.g. stage 4; stage 10+) are Type I, Type III and Type IV (Manasek, 1975) (see Section 5.3.2).

cribed in detail in Section 4.2. However, in the present experiments 0,2% hyaluronidase (Sigma H-3506) or 0,2% collagenase (Sigma C-6885 or C-3267) were included in the culture medium, as indicated in the experiments below. The hyaluronidase and collagenase stock solutions, prepared as 40mg per ml in previously autoclaved double-distilled water, were re-sterilized by filtration through 0,22µm millipore filters, and stored frozen.

5.3 Experiments and Results

5.3.1 An investigation of the effects of the removal of hyaluronate (by hyaluronidase treatment) on mesodermal erythropoiesis

The development of the yolk sac vascular system in the chick astoderm may be divided into two main phases: an initial phase involving the migration of the mesenchymal cells from the primitive streak into the AOV[1], followed by a second phase involving the overt differentiation of these cells into the various components of the yolk sac vascular system, viz., blood cells, endothelium, coelomic linings (see Chapter Two). This bi-phasic type of development is also characteristic of other systems, such as the development of the limb, axial skeleton, cornea, endocardium, limb regenerate, and the neural crest cells (Zwilling, 1968; Toole and Gross, 1971; Toole and Trelstad, 1971; Toole, 1972, 1973a; Markwald *et al*, 1975, 1977; Pratt *et al*, 1975, 1978; Toole *et al*, 1977; Derby, 1978; Derby *et al*, 1978; Orkin and Toole, 1978; Pintar, 1978). In all these systems there is a period of cell migration followed by a period of cell differentiation.

Toole and coworkers, and Pratt, Derby and coworkers, have reported a close correlation between hyaluronate synthesis and cell migration, and between hyaluronidase activity (i.e., hyaluronate removal) and cell differentiation in these latter systems. Furthermore, the phase of cell migration is often found to be associated with an increase in the volume of the extracellular spaces, believed to be due to the hydration

---

1. For a more extensive discussion of the migration of these primary mesenchymal cells, and the migration of sheets of cells in general, see Section 2.4.2.

of the hyaluronate molecules themselves (Toole and Gross, 1971; Toole and Trelstad, 1971; Toole *et al*, 1972, 1977; Pratt *et al*, 1975; Fisher and Solursh, 1977). In this regard, Fisher and Solursh (1977) have clearly demonstrated a correlation between the presence of hyaluronate and the increase in volume of the extracellular spaces during the migration of the primary mesenchymal cells from the primitive streak in the early chick blastoderm. Fisher and Solursh (1977) have further shown that the *in vivo* removal of hyaluronate with hyaluronidase results in a large decrease in the volume of the extracellular spaces, and the clumping together of the mesodermal cells.

In terms of these findings, it is pertinent to note that the first overt sign of yolk sac vasculogenesis is the clumping together of some of the mesodermal cells to form the blood-islands (see Section 2.3.2). It is tempting to suggest that the removal of hyaluronate, and the resultant decrease in volume of the extracellular spaces, may generate favourable conditions for the clumping together of the presumptive blood-island cells. Indeed, Toole and coworkers (Toole, 1972, 1973a; Toole *et al*, 1972, 1977) believe that the presence of hyaluronate may actually suppress cell differentiation.

The aim of the present study was therefore to investigate whether the removal of hyaluronate (by hyaluronidase treatment) has an effect on mesodermal erythropoiesis. Furthermore, in view of the belief of Toole and coworkers that hyaluronate may suppress cell differentiation (Toole, 1972, 1973; Toole *et al*, 1972, 1977), part of the present study has been focused also on the question of whether the inhibitory effect of the ectoderm on mesodermal erythropoiesis (as found in Chapter Four) might be mediated by hyaluronate itself. In this regard, Pratt *et al* (1978), using ^{3}H-glucosamine incorporation studies to investigate the source and distribution of hyaluronate during the migration of the chick cranial neural crest cells, found that hyaluronate is synthesized and secreted by the surface ectodermal cells of the early chick embryo. If hyaluronate were likewise being produced by the ectoderm of the yolk sac, and furthermore, if it indeed suppressed cell differentiation (as proposed by Toole and coworkers), then the removal of hyaluronate (by hyaluronidase treatment) should result in the enhancement of the erythropoietic differentiation of the ectoderm-plus-mesodermal explants. The present study therefore also investigates whether the removal of hyalur-

onate (by hyaluronidase treatment) counteracts the inhibitory influence exerted by the ectoderm on mesodermal erythropoiesis.

In this study the coverslip culture method has been used, and a comparison has been made of the erythropoietic differentiation of the following explants when cultured either in the presence or the absence of 0,2% hyaluronidase[1]:

Expt 5.I : meso (AOV, st 4);
Expt 5.II : meso + endo (AOV, st 8-9);
Expt 5.III : ecto + meso (AOV, st 4);
Expt 5.IV : ecto + meso (AP, st 4);
Expt 5.V : ecto + meso (AP, st 8-9).

As described below, it is found that the removal of hyaluronate by means of 0,2 hyaluronidase has no effect on the erythropoietic differentiation of the explants, and furthermore, that the removal of hyaluronate does not appear to affect the inhibitory influence exerted by the ectoderm on mesodermal erythropoiesis.

As part of every culture experiment, control explants isolated from a normally erythropoietic part of the blastoderm were routinely cultured and stained for haemoglobin (see Experiment 4.I, page 173). These controls were carried out to ensure that the culture medium and the o-dianisidine stain were always optimal.

The details of the experiments performed and the results obtained are presented below.

5.3.1.1 Culture experiments

Experiment 5.I (meso (AOV, st 4))

In this experiment the effect of 0,2 hyaluronidase treatment (i.e., hyaluronate removal) on the erythropoietic differentiation of the isolated stage 4 opaca mesoderm was investigated. 0,5mm x 0,5mm fragments of the stage 4 opaca mesoderm were isolated from the posterio

---

1. The hyaluronidase used (Sigma H-3506) is hyaluronidase Type I-S, a lyophilized powder prepared by the Sigma Chemical Company and specified as being approximately 300 NF units per mg solid (Sigma Price List Catalogue, February 1981, page 338).

lateral region of the AOV, as shown in Fig. 5.1, and were cultured for 72 hours in small drops of medium to which 0,2% hyaluronidase had been added.

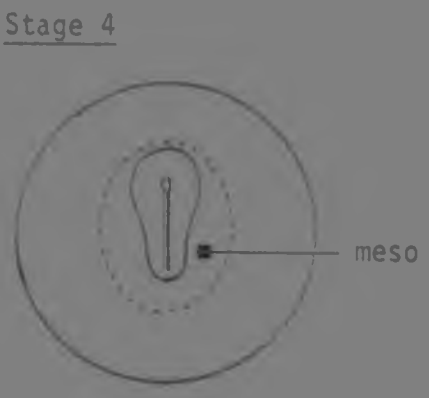


Figure 5.1

Diagram illustrating the area from where mesodermal fragments were isolated from the AOV of stage 4 blastoderms.

The erythropoietic differentiation of the explants cultured in the presence of 0,2% hyaluronidase was then compared with the erythropoietic differentiation of the same type of explants (i.e., stage 4 opaca mesoderm) when cultured in medium without added hyaluronidase. The following results were obtained, as tabulated in Table 5.1.

When the stage 4 opaca mesodermal fragments were cultured for 72 hours in medium containing 0,2% hyaluronidase,

-- 9,1% of the explants (1 case out of 11) exhibited traces of pale yellow staining (i.e., uncertain haemoglobin staining);

\+ 9,1% of the explants (1 case out of 11) exhibited pale brown (i.e., pale haemoglobin) staining in a large number of cells;

++ 45,4% of the explants (5 cases out of 11) exhibited more intense haemoglobin staining in a large number of cells;

+++ 36,4% of the explants (4 cases out of 11) exhibited very intense haemoglobin staining in a large number of cells.

These results are very comparable with the degree of erythropoiesis observed when the stage 4 opaca mesodermal fragments were cultured for 72 hours in medium without added hyaluronidase, as tabulated

hereunder.

-+ 3,6 of the explants (1 case out of 28) exhibited traces of pale yellow staining (i.e., uncertain haemoglobin staining);

+ 17,9% of the explants (5 cases out of 28) exhibited pale brown (i.e., pale haemoglobin) staining in a large number of cells;

++ 50% of the explants (14 cases out of 28) exhibited more intense haemoglobin staining in a large number of cells;

+++ 28,6 of the explants (8 cases out of 28) exhibited very intense haemoglobin staining in a large number of cells.

Indeed, it was found that there was no statistically significant difference between the erythropoietic differentiation of the stage 4 opaca mesodermal fragments when they were cultured in the presence or absence of added hyaluronidase (p = greater than 0,5). This therefore indicates that the erythropoietic differentiation of the stage 4 opaca mesoderm is not affected by the removal of hyaluronate (by hyaluronidase treatment).

A similar finding was made in the next experiment in which the effect of 0,2 hyaluronidase treatment on the erythropoietic differentiation of stage 8-9 opaca mesoderm-plus-endodermal fragments was investigated (Experiment 5.II).

Experiment 5.II (meso + endo (AOV, st 8-9))

Fragments composed of the stage 8-9 opaca mesoderm-plus-endoderm were isolated from the posterio-lateral AOV (Fig. 5.2) and cultured for 72 hours in small drops of medium which either contained 0,2 hyaluronidase or were free of added hyaluronidase.

The extent of erythropoiesis of the explants cultured in the presence or absence of added hyaluronidase was then compared. The following results were obtained, as tabulated in Table 5.1.

When the stage 8-9 opaca mesoderm-plus-endodermal fragments were cultured for 72 hours in medium containing 0,2% hyaluronidase,

++ 16,7 of the explants (2 cases out of 12) exhibited fairly intense haemoglobin staining in a large number of cells;

+++ 83,3 of the explants (10 cases out of 12) exhibited very intense haemoglobin staining in a large number of cells.

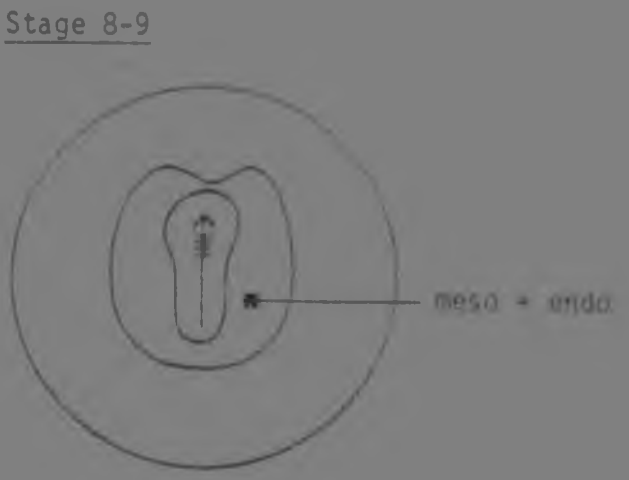


Figure 5.2

Diagram illustrating the area from where mesoderm-plus-endodermal fragments were isolated from the AOV of stage 8-9 blastoderms.

These results were very comparable with the extent of erythropoiesis observed when the stage 8-9 opaca mesoderm-plus-endodermal fragments were cultured for 72 hours in medium without added hyaluronidase, as tabulated below.

\+ 3,8% of the explants (1 case out of 26) exhibited pale brown (i.e., pale haemoglobin) staining in a large number of cells;

++ 19,2% of the explants (5 cases out of 26) exhibited more intense haemoglobin staining in a large number of cells;

+++ 76,9% of the explants (20 cases out of 26) exhibited very intense haemoglobin staining in a large number of cells.

Indeed, it was found that the erythropoietic differentiation of the stage 8-9 opaca mesoderm-plus-endoderm in the presence of 0,2% hyaluronidase was not significantly different from that of the explants cultured in medium without added hyaluronidase (p = greater than 0,5). Thus, the results of the present experiment (like those of the previous experiment in which the stage 4 opaca mesoderm was cultured in the presence or absence of added hyaluronidase) indicate that the erythropoietic differentiation of the stage 8-9 opaca mesoderm-plus-endodermal fragments is likewise not affected by the removal of hyaluronate (by 0,2% hyaluronidase treatment).

In the next three experiments the effect of hyaluronidase treatment

on the erythropoietic differentiation of explants composed of the pellucida- or opaca ectoderm-plus-mesoderm was investigated (Experiments 5.III to 5.V). The question being investigated was whether hyaluronate plays any role in the inhibitory effect of the ectoderm on mesodermal erythropoiesis, as observed in the experiments of the previous chapter (see Chapter Four). The present study was carried out in view of the belief of Toole and coworkers (Toole, 1972, 1973a; Toole *et al*, 1972, 1977) that hyaluronate may suppress cell differentiation, and further, in view of the findings of Pratt *et al* (1978) that hyaluronate may be secreted by the ectodermal cells themselves. The present study aimed to investigate whether the removal of hyaluronate (by hyaluronidase treatment) would reduce the inhibitory influence exerted by the ectoderm on mesodermal erythropoiesis. However, as detailed below, it is found that the removal of hyaluronate with 0,2% hyaluronidase treatment does not affect the inhibitory influence exerted by the ectoderm on mesodermal erythropoiesis.

Experiment 5.III (ecto + meso (AOV, st 4))

In this experiment the effect of 0,2% hyaluronidase treatment on the erythropoietic differentiation of stage 4 opaca ectoderm-plus-mesodermal explants was investigated. 0,5mm x 0,5mm fragments composed of ectoderm-plus-mesoderm were isolated from the AOV of stage 4 blastoderms (Fig. 5.3) and were cultured for 72 hours in small drops of medium to which 0,2% hyaluronidase had been added.

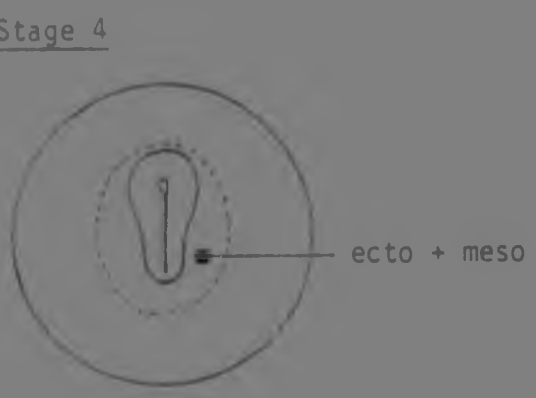


Figure 5.3

Diagram illustrating the area from where ectoderm-plus-mesodermal fragments were isolated from the AOV of stage 4 blastoderms.

The erythropoietic differentiation of the explants cultured in the presence of 0,2% hyaluronidase was then compared with the erythropoietic differentiation of the same type of explants (i.e., stage 4 opaca ectoderm-plus-mesoderm) when cultured in medium without added hyaluronidase. The following results were obtained, as tabulated in Table 5.1.

When the stage 4 opaca ectoderm-plus-mesodermal fragments were cultured for 72 hours in medium containing 0,2% hyaluronidase,

- 28,6% of the explants (4 cases out of 14) stained the blue colour of the counterstain only (i.e., the staining for haemoglobin was negative);

-+ 21,4% of the explants (3 cases out of 14) exhibited traces of pale yellow staining (i.e., uncertain haemoglobin staining);

+ 21,4% of the explants (3 cases out of 14) exhibited pale brown (i.e., pale haemoglobin) staining in a small number of cells;

++ 28,6% of the explants (4 cases out of 14) exhibited more intense haemoglobin staining in a small number of cells.

These results are very comparable with the extent of erythropoiesis observed when the stage 4 opaca ectoderm-plus-mesodermal fragments were cultured for 72 hours in medium without added hyaluronidase, as tabulated hereunder.

- 35% of the explants (7 cases out of 20) stained the blue colour of the counterstain only (i.e., the staining for haemoglobin was negative);

-+ 30% of the explants (6 cases out of 20) exhibited traces of pale yellow staining (i.e., uncertain haemoglobin staining);

+ 20% of the explants (4 cases out of 20) exhibited pale brown (i.e., pale haemoglobin) staining in a small number of cells;

++ 15% of the explants (3 cases out of 20) exhibited more intense haemoglobin staining in a small number of cells.

Indeed, it was found that there was no significant difference between the erythropoietic differentiation of the stage 4 opaca ectoderm-plus-mesodermal explants when cultured in the presence or absence of added hyaluronidase (p = greater than 0,2). This indicates that the removal of hyaluronate (with hyaluronidase treatment) does not remove the inhibitory effect of the opaca ectoderm on mesodermal erythropoiesis.

This would suggest that other factors, rather than hyaluronate, must be responsible for the inhibitory influence of the stage 4 opaca ectoderm on mesodermal erythropoiesis.

The same type of result was obtained in the next experiment in which the effects of hyaluronate removal on the inhibitory influence of the stage 4 pellucida ectoderm was investigated (Experiment 5.IV).

Experiment 5.IV (ecto + meso (AP, st 4))

Fragments composed of the stage 4 pellucida ectoderm-plus-mesoderm (Fig. 5.4) were isolated and cultured for 72 hours in small drops of medium to which 0,2% hyaluronidase had been added.

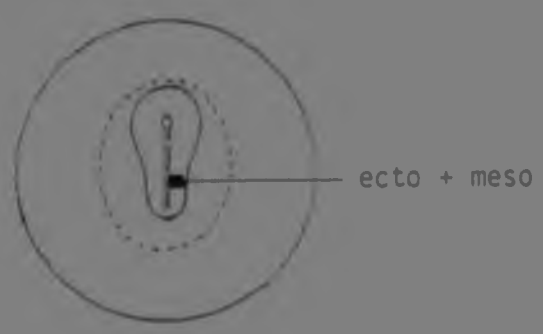


Figure 5.4

Diagram illustrating the area from where ectoderm-plus-mesodermal fragments were isolated from the AP of stage 4 blastoderms.

The erythropoietic differentiation of the explants cultured in the presence of 0,2% hyaluronidase was then compared with the erythropoietic differentiation of the same type of explants (i.e., stage 4 pellucida ectoderm-plus-mesoderm) which had been cultured in medium without added hyaluronidase. The following results were obtained, as tabulated in Table 5.1.

When the stage 4 pellucida ectoderm-plus-mesodermal fragments were cultured for 72 hours in medium containing 0,2% hyaluronidase, 16,7% of the explants (2 cases out of 12) stained the blue colour of the counterstain only (i.e., the staining for haemoglobin was

negative);

-+ 16,7% of the explants (2 cases out of 12) exhibited traces of pale yellow staining (i.e., uncertain haemoglobin staining);

+ 33,3% of the explants (4 cases out of 12) exhibited pale brown (i.e., pale haemoglobin) staining in a small number of cells;

++ 33,3% of the explants (4 cases out of 12) exhibited more intense haemoglobin staining in a small number of cells.

These results are very comparable with the degree of erythropoiesis observed when the stage 4 pellucida ectoderm-plus-mesodermal explants were cultured for 72 hours in medium without added hyaluronidase, as tabulated below.

- 30,4% of the explants (7 cases out of 23) stained the blue colour of the counterstain only (i.e., the staining for haemoglobin was negative);

-+ 17,4% of the explants (4 cases out of 23) exhibited traces of pale yellow staining (i.e., uncertain haemoglobin staining);

+ 21,8% of the explants (5 cases out of 23) exhibited pale brown (i.e., pale haemoglobin) staining in a small number of cells;

++ 30,4% of the explants (7 cases out of 23) exhibited more intense haemoglobin staining in a variable number of cells.

Indeed, it was found that there was no statistically significant difference between the degree of erythropoiesis observed when the stage 4 pellucida ectoderm-plus-mesodermal explants were cultured in the presence or absence of added hyaluronidase (p = greater than 0.5). Thus, the removal of hyaluronate by means of hyaluronidase treatment does not appear to affect the inhibitory influence of the stage 4 pellucida ectoderm on mesodermal erythropoiesis. This indicates that the inhibitory effect of the stage 4 pellucida ectoderm (like that of the stage 4 opaca ectoderm) is not due to the presence of hyaluronate.

A similar result was obtained in the next experiment in which the effect of hyaluronate removal on the erythropoietic differentiation of the ectoderm-plus-mesodermal explants from the stage 8-9 AP was investigated (Experiment 5.V).

Experiment 5.V (ecto + meso (AP, st 8-9))

Fragments composed of the stage 8-9 pellucida ectoderm-plus-

mesoderm (Fig. 5.5) were isolated and cultured for 72 hours in small drops of medium to which 0,2% hyaluronidase had been added.

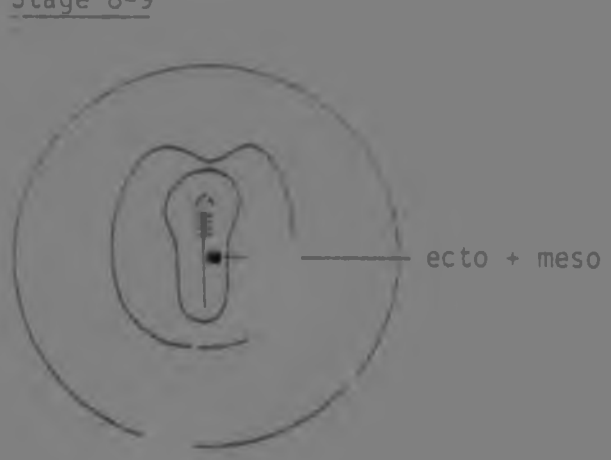


Figure 5.5

Diagram illustrating the area from where ectoderm-plus-mesodermal fragments were isolated from the AP of stage 8-9 blastoderms.

The erythropoietic differentiation of the explants cultured in the presence of hyaluronidase was then compared with the extent of erythropoiesis of the same type of explants which had been cultured in medium without added hyaluronidase. The following results were obtained, as tabulated in Table 5.1.

- 84,6% of the explants (11 cases out of 13) stained the blue colour of the counterstain only (i.e., the staining for haemoglobin was negative);
-+ 7,7% of the explants (1 case out of 13) exhibited traces of pale yellow staining (i.e., uncertain haemoglobin staining);
+ 7,7% of the explants (1 case out of 13) exhibited pale brown (i.e., pale haemoglobin) staining in a small number of cells.

These results are very comparable with the extent of erythropoiesis observed when the stage 8-9 pellucida ectoderm-plus-mesodermal explants were cultured for 72 hours in medium without added hyaluronidase, as tabulated hereunder.

(Text continues on page 256).

Table 5.1 Table summarizing the results of the study to investigate the effects of the removal of hyaluronate (by 0,2% hyaluronidase* treatment) on mesodermal erythropoiesis.

Experiments	Types of Explants	Hyaluronidase Treatment*	Total No. of Explants	No. of Explants (Categories of Haemoglobin Staining (See Key, Fig. 4.1 page 171))					% of Total No.				
				-	-+	+	++	+++	-	-+	+	++	+++
Expt. 5.I	meso (AOV, st 4)	Yes	11	0	1	1	5	4	0,0	9,1	9,1	45,4	36,4
		No	28	0	1	5	14	8	0,0	3,6	17,9	50,0	28,6
Expt. 5.II	meso + endo (AOV, st 8-9)	Yes	12	0	0	0	2	10	0,0	0,0	0,0	16,7	83,3
		No	26	0	0	1	5	20	0,0	0,0	3,8	19,2	76,9
Expt. 5.III	ecto + meso (AOV, st 4)	Yes	14	4	3	3	4	0	28,6	21,4	21,4	28,6	0,0
		No	20	7	6	4	3	0	35,0	30,0	20,0	15,0	0,0
Expt. 5.IV	ecto + meso (AP, st 4)	Yes	12	2	2	4	4	0	16,7	16,7	33,3	33,3	0,0
		No	23	7	4	5	7	0	30,4	17,4	21,8	30,4	0,0
Expt. 5.V	ecto + meso (AP, st 8-9)	Yes	13	11	1	1	0	0	84,6	7,7	7,7	0,0	0,0
		No	20	18	2	0	0	0	90,0	10,0	0,0	0,0	0,0
Expt. 4.I (Routine Control)	ecto + meso + endo (AOV, st 8 9)	No	167	0	0	0	0	167	0,0	0,0	0,0	0,0	100,0

*The hyaluronidase is Sigma H-3506

- 90% of the explants (18 cases out of 20) stained the blue colour of the counterstain only (i.e., the staining for haemoglobin was negative);

-+ 10% of the explants (2 cases out of 20) exhibited traces of pale yellow staining (i.e., uncertain haemoglobin staining).

Indeed, it was found that there was no statistically significant difference between the extent of erythropoiesis exhibited by the stage 8-9 pellucida ectoderm-plus-mesodermal explants when they were cultured in the presence or absence of added hyaluronidase (p = greater than 0,2). Thus, hyaluronate does not appear to affect the extent of erythropoiesis of the stage 8-9 pellucida ectoderm-plus-mesodermal fragments.

Summarizing the results of the present investigation in which the effect of hyaluronidase treatment on mesodermal erythropoiesis was investigated, it appears that the removal of hyaluronate, by means of 0,2% hyaluronidase treatment, has no effect on the erythropoietic differentiation of the mesodermal cells. Furthermore, the treatment of explants composed of ectoderm-plus-mesoderm with 0,2% hyaluronidase does not appear to affect the inhibitory influence exerted by the ectoderm on mesodermal erythropoiesis. Thus, the presence of hyaluronate does not appear to inhibit mesodermal erythropoiesis, or to be responsible for the inhibitory effect of the ectoderm on mesodermal erythropoiesis.

A relevant point noted in the present study is that the erythropoietic differentiation of the hyaluronidase-treated explants always tended to be either equal to, or slightly better than, that of the explants cultured in the absence of added hyaluronidase (i.e., the erythropoietic differentiation of the hyaluronidase-treated explants was never inferior to that of the untreated explants - see Table 5.1). However, these differences are not statistically significant.

5.3.2 An investigation of the effects of the removal of collagen (by collagenase treatment) on mesodermal erythropoiesis

Manasek (1975), using ^{3}H-proline incorporation to investigate the synthesis of collagen in the AP and AOV of the early chick blastoderm, found that collagens Type I, Type III and Type IV are being synthesized in the stage 4 and stage 8+ to 11+ chick blastoderm, the predominant species being Type I.

Collagens Type I, Type II, Type III and Type IV are four different types of collagen which occur in different tissues (for reviews, see Miller and Matukas, 1974; Miller, 1977). Type I collagen is a major constituent of all the major connective tissues, e.g., skin, bone, tendon and dentin. Type II collagen is found only in hyaline cartilage (Miller, 1971) and in the notochord (Linsenmayer *et al*, 1973; Miller and Mathews, 1974). Type III collagen is prevalent in selected connective tissues such as dermis, major blood vessels, and the uterine wall. Type IV collagen is the collagenous component of basement membranes (Kefalides, 1971).

Type I collagen molecules are built up from two $\alpha 1(I)$ chains and one $\alpha 2$ chain, coiled into a triple helical molecule $\left[\alpha 1(I)\right]_2\alpha 2$. Type II and Type III collagen molecules are each composed of three identical chains, and have molecular formulae $\left[\alpha 1(II)\right]_3$ and $\left[\alpha 1(III)\right]_3$, respectively. There is at present still disagreement concerning both the number of distinct collagens which comprise basement membrane (Type IV) collagen, and the nature of their constituent collagen chains (see Rhodes and Miller, 1978; Kresina and Miller, 1979). The different types of collagen chains which make up the different collagen types e.g., $\alpha 1(I)$, $\alpha 2$, etc., are genetically distinct from each other and differ in amino acid composition (see Miller and Matukas, 1974; Miller, 1977).

Manasek (1975) found that the level of collagen synthesized in the early, pre-connective tissue stage chick blastoderm (stage 4, stage 8+ to 11+) is very much lower than that observed in mature connective tissue. Indeed, collagen is found to comprise only about 0,2% of the proline-labelled proteins synthesized by stage 4 blastoderms, and only 5-10% of the proline-labelled proteins synthesized by stage 8+ to 11+ blastoderms. This suggests that collagen is only a minor component of these early embryonic extracellular matrices (Manasek, 1975).

In this regard, Murphy and Carlson (1978), in an ultrastructural study of the extracellular matrix of the developing yolk sac vascular system itself, reported the conspicuous absence of extracellular matrix materials such as collagen, basement membranes and amorphous materials within the developing yolk sac blood system during the early stages of

vasculogenesis, up to stage 11 (13 somites; 40-45 hours incubation). Thus, apart from the basement membranes adjacent to both the ectoderm and endoderm, which are laid down during the stages of primitive streak formation (Trelstad et al., 1967; Hay, 1968, 1973), no extracellular matrix materials are visible by TEM within the developing vessels until stage 11. This is the stage at which the first blood cells are already becoming liberated into the vessel lumens (see Chapter Two). This indicates that the collagenous extracellular matrix materials of the yolk sac blood system itself are not laid down until after the erythropoietic differentiation of the mesodermal cells is already well underway. However, from stage 11 onwards, numerous 15nm microfibrils become observable between the vessel endothelium and the splanchnopleure, and by stage 15 (24-27 somites; 50-55 hours incubation) a scanty discontinuous basement membrane is seen between the vessel endothelium and the splanchnopleure (Murphy and Carlson, 1978). Collagen[1] fibrils are first observable between the endothelium and the splanchnopleure at stage 20 (40-43 somites; 70-72 hours incubation), when the endothelium already resembles mature vascular endothelium, and the adjacent splanchnopleure is developing into vascular smooth muscle (Murphy and Carlson, 1978).

In the present study the effect of the removal of collagen (by collagenase treatment) on mesodermal erythropoiesis has been investigated. The coverslip culture method has been used, and a comparison has been made of the erythropoietic differentiation of the following explants when cultured in the presence or the absence of 0,2% collagenase[2].

Expt 5.VI : meso (AOV, st 4);
Expt 5.VII : meso + endo (AOV, st 8-9);
Expt 5.VIII : ecto + meso + endo (AOV, st 8-9).

In the initial experimental runs the collagenase used (Sigma C-6885) was 'impure' in that it is not specified to be free of non-

---

1. This is probably collagen Type IV (basement membrane collagen). However, Murphy and Carlson (1978) make no mention of collagen type.

2. See footnotes overleaf.

specific protease activity[1]. In the later experimental runs a more highly purified collagenase preparation (Sigma C-3267) was used, which is specified to be essentially free of non-specific protease activity[2]. In each experiment the erythropoietic differentiation of the explants cultured in the presence of the 'impure' and 'pure' collagenases has been compared with that of the explants cultured in the absence of added collagenase.

It was found that:

1. The addition of 0,2% 'pure' collagenase (Sigma C-3267) to the culture medium did not affect the erythropoietic differentiation of the stage 4 opaca mesodermal fragments or the stage 8-9 opaca ectoderm-plus-mesoderm-plus-endodermal explants. However, it caused a significant reduction in the erythropoietic differentiation of the stage 8-9 opaca mesoderm-plus-endodermal explants.
2. The addition of 0,2% 'impure' collagenase (Sigma C-6885) to the culture medium caused a significant reduction, or total suppression, in the erythropoietic differentiation of the blastoderm explants.
3. In most instances the treatment of the explants with collagenase was accompanied by a loss of tissue integrity during the culturing period.

The details of the experiments performed and the results obtained are outlined below, and summarized in Table 5.2.

5.3.2.1 Culture experiments

Experiment 5.VI (meso(AOV, st 4))

In this experiment the effect of 0,2% collagenase treatment (i.e., collagen removal) on the erythropoietic differentiation of the isolated stage 4 opaca mesoderm was investigated. 0,5mm x 0,5mm fragments of the stage 4 opaca mesoderm were isolated from the posterio -lateral AOV

---

1. The 'impure' collagenase used (Sigma C-6885) is collagenase Type II (approximate activity, 125-200 units per mg solid), prepared by Sigma and specified as being low in tryptic activity relative to general use collagenase. However, this collagenase is not specified to be free of non-specific protease or clostripain activities (Sigma Price List Catalogue, February 1981, page 202).
2. The 'pure' collagenase used (Sigma C-3267) is designated by Sigma as being high purity collagenase. This collagenase is a chromatographically purified and lyophilized Type VI collagenase (activity, 600-900 units per mg solid), prepared by Sigma and specified as being substantially free of non-specific protease, clostripain and tryptic activities (Sigma Price List Catalogue, February 1981, page 202).

(Fig. 5.6) and cultured for 72 hours in small drops of medium to which 0,2% collagenase had been added. In the initial runs the collagenase preparation used was 'impure' in that it was not specified to be free of non-specific protease or clostripain activities (Sigma C-6885). In the later runs the more highly purified collagenase preparation (Sigma C-3267) was used.

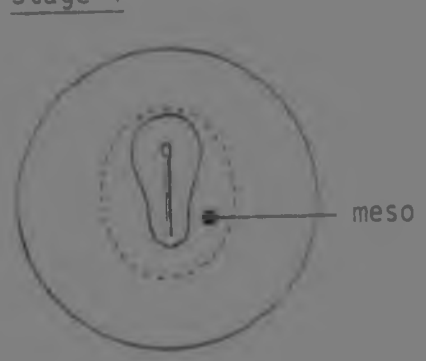


Figure 5.6

Diagram illustrating the area from where mesodermal fragments were isolated from the AOV of stage 4 blastoderms.

The erythropoietic differentiation of the explants cultured in the presence of 0,2% collagenase was then compared with the erythropoietic differentiation of the same type of explants (i.e., stage 4 opaca mesoderm) when cultured in medium without added collagenase. The following results were obtained, as tabulated in Table 5.2.

When the stage 4 opaca mesodermal fragments were cultured for 72 hours in medium containing 0,2% 'impure' collagenase (Sigma C-6885), 100% of the explants (10 cases out of 10) stained the blue colour of the counterstain only (i.e., all explants exhibited negative staining for haemoglobin). Thus, the presence of 0,2% 'impure' collagenase (Sigma C-6885) results in a total suppression of haemoglobin synthesis by the stage 4 opaca mesodermal explants.

This in contrast to the situation which occurred when the stage 4 opaca mesoderm was cultured for 72 hours in medium without added collagenase, in which case the following results were obtained.

-+ 3,6% of the explants (1 case out of 28) exhibited traces of pale yellow staining (i.e., uncertain haemoglobin staining);

+ 17,9% of the explants (5 cases out of 28) exhibited pale brown (i.e., pale haemoglobin) staining in a large number of cells;

++ 50% of the explants (14 cases out of 28) exhibited more intense haemoglobin staining in a large number of cells;

+++ 28,6% of the explants (8 cases out of 28) exhibited very intense haemoglobin staining in a large number of cells.

When, in the other hand, the stage 4 opaca mesodermal explants were cultured in the presence of 0,2% 'pure' collagenase (Sigma C-3267), the extent of erythropoiesis was found to be very comparable with that of the same type of explants cultured in medium without added collagenase. As tabulated in Table 5.2, when the stage 4 opaca mesodermal fragments were cultured in medium to which 0,2% 'pure' collagenase had been added (Sigma C-3267),

+ 42,9% of the explants (6 cases out of 14) exhibited pale brown (i.e., pale haemoglobin) staining in a large number of cells;

++ 28,6% of the explants (4 cases out of 14) exhibited more intense haemoglobin staining in a large number of cells;

+++ 28,6% of the explants (4 cases out of 14) exhibited very intense haemoglobin staining in a large number of cells.

Indeed, it was found that there was not a statistically significant difference between the extent of erythropoiesis exhibited by the stage 4 opaca mesodermal fragments when they were cultured in the presence or absence of the highly purified collagenase (Sigma C-3267) (p = greater than 0,5).

Thus, whereas the presence of 0,2% 'impure' collagenase (Sigma C-6885) results in a total suppression in the erythropoietic differentiation of the stage 4 opaca mesodermal explants, the presence of 0,2% 'pure' collagenase (Sigma C-3267) does not suppress the erythropoietic differentiation of the stage 4 opaca mesodermal explants.

An interesting point noted in the present study was that when the stage 4 opaca mesodermal explants were treated with 0,2% collagenase (either 'pure' or 'impure'), the explants tended to lose their tissue integrity during the culturing period, and take on the appearance of a mass of cells, loosely aggregated together. This loss of tissue

integrity was clearly evident when the tissue was fixed, since the mass of mesodermal cells was seen to become totally dispersed as soon as the tissue was disturbed by the convection currents set up in the medium by the addition of the fixative to the medium. It is of interest to note that this loss of tissue integrity occurred both in the explants treated with the 'impure' collagenase (Sigma C-6885), in which the erythropoietic differentiation was totally suppressed, and in the explants treated with 'pure' collagenase (Sigma C-3267), in which the erythropoietic differentiation was not affected.

Experiment 5.VII (meso + endo (AOV, st 8-9))

In this experiment, the effect of 0,2% collagenase treatment (i.e., collagen removal) on the erythropoietic differentiation of stage 8-9 opaca mesoderm-plus-endodermal explants was investigated. 0,5mm x 0,5mm fragments composed of the stage 8-9 mesoderm-plus-endoderm were isolated from the posterio-lateral AOV (Fig. 5.7), and cultured for 72 hours in small drops of medium to which 0,2% collagenase had been added. In the initial experimental runs the 'impure' type of collagenase (Sigma C-6885) was used. In the later runs the more highly purified collagenase (Sigma C-3267) was used.

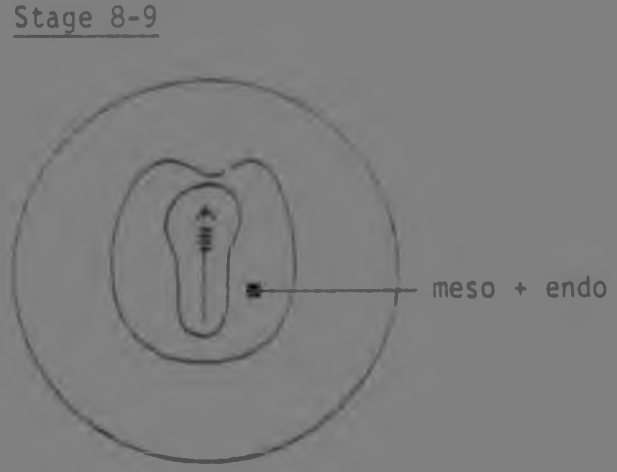


Figure 5.7

Diagram illustrating the area from where mesoderm-plus-endodermal fragments were isolated from the AOV of stage 8-9 blastoderms.

The erythropoietic differentiation of the explants cultured in the presence of 0,2% collagenase was then compared with that of the same type of explants (i.e., the stage 8-9 opaca mesoderm-plus-endoderm) when cultured in medium without added collagenase. The following results were obtained, as tabulated in Table 5.2.

When the stage 8-9 opaca mesoderm-plus-endodermal explants were cultured for 72 hours in medium containing 0,2% 'impure' collagenase (Sigma C-6885), there was a highly significant suppression in the erythropoietic differentiation of the explants compared to when they were cultured in medium without added collagenase (p = very much less than 0,001). Indeed, in the presence of 0,2% 'impure' collagenase (Sigma C-6885),

- 80% of the explants (8 cases out of 10) stained [illegible]ue colour of the counterstain only (i.e., the staining f[illegible] haemoglobin was negative);
-+ 20% of the explants (2 cases out of 10) exhibited traces of pale yellow staining (i.e., uncertain haemoglobin staining).

Thus, the synthesis of haemoglobin by the stage 8-9 opaca mesoderm-plus-endodermal explants in the presence of 0,2% 'impure' collagenase (Sigma C-6885) is almost totally non-existent. On the other hand, when the stage 8-9 opaca mesoderm-plus-endodermal explants were cultured in medium without added collagenase,

+ 3,8% of the explants (1 case out of 26) exhibited pale brown (i.e., pale haemoglobin) staining in a large number of cells;
++ 19,2% of the explants (5 cases out of 26) exhibited more intense haemoglobin staining in a large number of cells;
+++ 76,9% of the explants (20 cases out of 26) exhibited very intense haemoglobin staining in a large number of cells.

When, however, the stage 8-9 opaca mesoderm-plus-endodermal explants were cultured for 72 hours in the presence of 'pure' collagenase (Sigma C-3267), their erythropoietic differentiation, although significantly greater than that of the explants cultured in the presence of the 'impure' collagenase (Sigma C-6885) (p = less than 0,001), was nevertheless still significantly lower than that of the explants cultured in the absence of collagenase (p = less than 0,005). As tabulated in Table 5.2, when the stage 8-9 opaca mesoderm-plus-endodermal fragments

were cultured in medium containing 0,2% 'pure' collagenase (Sigma C-3267),

\+ 53,3% of the explants (8 cases out of 15) exhibited pale brown (i.e., pale haemoglobin) staining in a variable number of cells;

++ 6,7% of the explants (1 case out of 15) exhibited more intense haemoglobin staining in a large number of cells;

+++ 40% of the explants (6 cases out of 15) exhibited very intense haemoglobin staining in a large number of cells.

Thus, whereas the presence of 0,2% 'impure' collagenase (Sigma C-6885) almost totally suppresses the erythropoietic differentiation of the stage 8-9 opaca mesoderm-plus-endodermal explants, the presence of 0,2% 'pure' collagenase (Sigma C-3267) results in a significant suppression in the erythropoietic differentiation of the same type of explants. Coupled with this finding, it was again found that the blastoderm fragments treated with 0,2% collagenase (both 'impure' and 'pure') tended to lose their tissue integrity during the culturing period.

A relevant point noted in the present study was that the extent of erythropoiesis exhibited by the stage 8-9 opaca mesoderm-plus-endodermal fragments in the presence of the 'pure' collagenase (Sigma C-3267) was very comparable with the extent of erythropoiesis exhibited by explants composed of the stage 4 opaca mesoderm cultured in medium containing the 'pure' collagenase (Sigma C-3267) or without added collagenase (see Experiment 5.VI) (p = greater than 0,5). It would thus appear that in the presence of the highly purified collagenase (Sigma C-3267) the erythropoietic differentiation of the stage 8-9 opaca mesoderm-plus-endodermal fragments is reduced to a level equal to that of the stage 4 opaca mesodermal fragments cultured in the absence of the opaca endoderm. This would suggest that the presence of 0,2% highly purified collagenase removes the salutary effect of the opaca endoderm on mesodermal erythropoiesis.

Experiment 5.VIII (ecto + meso + endo (AOV, st 8-9))

In this experiment the effect of 0,2% collagenase treatment on the erythropoietic differentiation of stage 8-9 opaca explants composed of all three germinal layers (i.e., ectoderm, mesoderm and endoderm) was investigated. 0,5mm x 0,5mm fragments composed of all three germinal layers were isolated from the posterio-lateral region of the stage 8-9

AOV (Fig. 5.8) and were cultured for 72 hours in small drops of medium to which 0,2% collagenase had been added. In the initial runs the 'impure' type of collagenase (Sigma C-6885) was used. In the later experimental runs the more highly purified collagenase preparation (Sigma C-3267) was used.

Stage 8-9

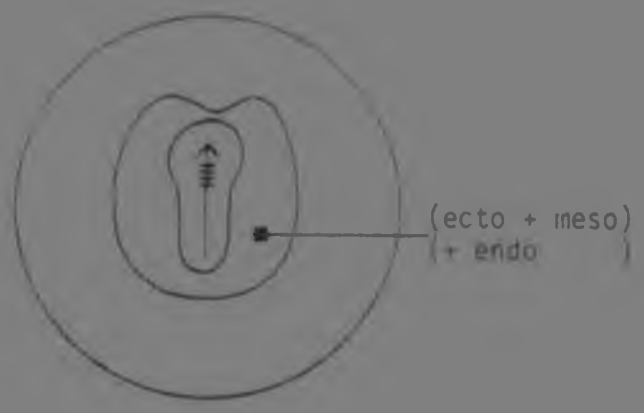


Figure 5.8

Diagram illustrating the area from where fragments composed of all three germinal layers (i.e., ectoderm, mesoderm and endoderm) were isolated from the AOV of stage 8-9 blastoderms.

The erythropoietic differentiation of the explants cultured in the presence of 0,2% collagenase was then compared with that of the same type of explants when cultured in medium without added collagenase. The following results were obtained, as tabulated in Table 5.2.

When the stage 8-9 opaca fragments composed of all three germinal layers were cultured for 72 hours in medium containing 0,2% 'impure' collagenase (Sigma C-6885),

- 22,7% of the explants (5 cases out of 22) stained the blue colour of the counterstain only (i.e., the staining for haemoglobin was negative);

-+ 4,5% of the explants (1 case out of 22) exhibited traces of pale yellow staining (i.e., uncertain haemoglobin staining);

+ 18,2% of the explants (4 cases out of 22) exhibited pale brown (i.e., pale haemoglobin) staining in a variable number of cells;

++ 13,6 of the explants (3 cases out of 22) exhibited more intense haemoglobin staining in a large number of cells;

+++ 40,9 of the explants (9 cases out of 22) exhibited very intense haemoglobin staining in a large number of cells.

These results are significantly lower than those of the same type of explants cultured in medium without added collagenase, in which case 100% of the explants (167 cases out of 167) were found to exhibit very intense haemoglobin staining in a large number of cells (p = much less than 0,001). Again, it was found that in the presence of the 'impure' collagenase (Sigma C-6885) the blastoderm fragments tended to lose their tissue integrity during the culturing period.

When, on the other hand, the explants were cultured in medium containing 0,2 highly purified collagenase (Sigma C-3267), the extent of erythropoiesis was as good as that of the explants cultured in the absence of added collagenase, i.e., 100 of the explants (12 cases out of 12) exhibited very intense haemoglobin staining in a large number of cells. Coupled with this observation it was found that the present explants in the presence of 0,2 'pure' collagenase (Sigma C-3267) were the only collagenase-treated explants which did not lose their tissue integrity during the culturing period. It could be suggested that these intact tissue explants composed of all three germinal layers may have hampered the penetration of the 'pure' collagenase through the tissue.

Summarizing the results of the present study in which the effect of collagenase treatment on mesodermal erythropoiesis was investigated, it is found that whereas treatment with 0,2 'impure' collagenase (Sigma C-6885) (i.e., collagenase which is not specified to be free of non-specific protease activity) causes a significant decrease or total suppression in mesodermal erythropoiesis, treatment with 0,2 highly purified collagenase (Sigma C-3267) does not affect the erythropoietic differentiation of the stage 4 opaca mesoderm itself, but may counteract the salutary effect of the opaca endoderm on mesodermal erythropoiesis. These effects are coupled with the loss of tissue integrity during the culturing period.

(Text continues on page 268).

Table 5.2 Table summarizing the results of the study to investigate the effects of the removal of collagen (by 0,2% collagenase* treatment) on mesodermal erythropoiesis.

Experiments	Types of Explants	Collagenase Treatment*	Total No. of Explants	Categories of Haemoglobin Staining (See Key, Fig. 4.1 page 171). No. of Explants					% of Total No.				
				-	-+	+	++	+++	-	-+	+	++	+++
Expt. 5.VI	meso (AOV, st 4)	'impure'	10	10	0	0	0	0	100,0	0,0	0,0	0,0	0,0
		'pure'	14	0	0	6	4	4	0,0	0,0	42,9	28,6	28,6
		no	28	0	1	5	14	8	0,0	3,6	17,9	50,0	28,6
Expt. 5.VII	meso + endo (AOV, st 8-9)	'impure'	10	8	2	0	0	0	80,0	20,0	0,0	0,0	0,0
		'pure'	15	0	0	8	1	6	0,0	0,0	53,3	6,7	40,0
		no	26	0	0	1	5	20	0,0	0,0	3,8	19,2	76,9
Expt. 5.VIII	ecto + meso + endo (AOV, st 8-9)	'impure'	22	5	1	4	3	9	22,7	4,5	18,2	13,6	40,9
		'pure'	12	0	0	0	0	12	0,0	0,0	0,0	0,0	100,0
		no	167	0	0	0	0	167	0,0	0,0	0,0	0,0	100,0

*The 'impure' collagenase is Sigma C-6885; the 'pure' collagenase is Sigma C-3267.

5.4 Discussion

In the present discussion, attention is focused first on the observations of the effect of hyaluronidase treatment on mesodermal erythropoiesis (Section 5.4.1), and then on the observations of the effect of collagenase treatment on mesodermal erythropoiesis (Section 5.4.2).

5.4.1 Discussion of the investigation of the effect of the removal of hyaluronate (by hyaluronidase treatment) on mesodermal erythropoiesis

The findings from the present study in which the effect of hyaluronidase treatment on mesodermal erythropoiesis has been investigated indicate that:

1. The removal of hyaluronate by means of 0,2% hyaluronidase treatment has no effect on the erythropoietic differentiation of the mesodermal cells, and
2. The removal of hyaluronate with 0,2% hyaluronidase does not appear to affect the inhibitory influence exerted by the ectoderm on mesodermal erythropoiesis.

These findings come from the observations of the extent of erythropoiesis in the following explants when cultured in the presence or absence of added hyaluronidase:

1. meso (AOV, st 4) = Expt 5.I;
2. meso + endo (AOV, st 8-9) = Expt 5.II;
3. ecto + meso (AOV, st 4) = Expt 5.III;
4. ecto + meso (AP, st 4) = Expt 5.IV;
5. ecto + meso (AP, st 8-9) = Expt 5.V.

In all cases it was found that the extent of erythropoiesis of the explants in the presence of 0,2% hyaluronidase was very comparable with that of the same type of explants cultured in medium without added hyaluronidase. Thus, the removal of hyaluronate did not appear to affect the erythropoietic differentiation of these explants.

These findings are in contrast to the views of Toole and coworkers, and to the findings of a number of other workers in the field, that hyaluronate may suppress cell differentiation (Toole *et al*, 1972, 1977;

Toole, 1973a; Wiebkin and Muir, 1973; Solursh *et al*, 1974; Muir, 1977). However, this view is not universally applicable, since Nevo and Dorfman (1972), Kosher *et al* (1973), Finch *et al* (1978) and Lash and Vasan (1978) did not find an inhibitory effect of hyaluronate on *in vitro* chondrogenesis, and Meier and Hay (1974b) reported no measurable effect of hyaluronate on glycosaminoglycan synthesis by embryonic corneal epithelial cells *in vitro*. Furthermore, Manasek *et al* (1973) reported the persistence of hyaluronate in the embryonic chick heart *in vivo* both during and after myocardial differentiation. These findings, and those of the present study, thus suggest that hyaluronate may not be a general inhibitor of cell differentiation.

The second observation from the present study, that the removal of hyaluronate (by hyaluronidase treatment) does not remove the inhibitory effect of the ectoderm (pellucida or opaca) on mesodermal erythropoiesis, suggests that factors other than hyaluronate must be responsible for the inhibitory influence of the ectoderm on mesodermal erythropoiesis (see Chapters Four and Six).

One criticism of the experimental design employed in the present investigation is that if hyaluronidase were produced by the blastoderm itself during the phase of overt differentiation[1], then the hyaluronate would anyway become removed even in the untreated explants. If this were the case, then the treatment of the explants with exogenous hyaluronidase in the present study would, at most, have speeded up the removal of the hyaluronate. An alternative, and probably better, method of investigating the effect of hyaluronate on mesodermal erythropoiesis would therefore have been that of adding hyaluronate itself to the cultures, and observing whether it suppressed mesodermal erythropoiesis.

5.4.2 Discussion of the investigation of the effect of the removal of collagen (by collagenase treatment) on mesodermal erythropoiesis

In investigating the effects of collagenase treatment (i.e., collagen removal) on mesodermal erythropoiesis, differences were observed in the

---

1. This refers to the belief of Toole and coworkers (Toole *et al*, 1972, 1977) and others that there is a close correlation between hyaluronidase activity and cell differentiation (see Section 5.3.1).

extent of erythropoiesis of the blastoderm explants when cultured (a) in the presence of a collagenase preparation which is not specified to be free of non-specific protease or clostripain activities, i.e., 'impure' collagenase (Sigma C-6885); (b) in the presence of a highly purified collagenase preparation which is specified to be essentially free of non-specific protease, clostripain and tryptic activities (Sigma C-3267); or (c) in a medium without added collagenase. This study involved a comparison of the extent of mesodermal erythropoiesis in the following explants when cultured under the above three conditions:

1. meso (AOV, st 4) = Expt 5.VI;
2. meso + endo (AOV, st 8-9) = Expt 5.VII;
3. ecto + meso + endo (AOV, st 8-9) = Expt 5.VIII.

The finding that the use of the 'impure' collagenase preparation (Sigma C-6885) caused a significant decrease, or total suppression, in the erythropoietic differentiation of all three types of blastoderm fragments would indicate that the low-level contamination of the collagenase preparation with non-specific protease and clostripain activities is sufficient to block cell differentiation. As this 'impure' collagenase treatment did not appear to break down the cells themselves (i.e., cell lysis or pycnosis was not observed), it is probable that the protease contaminant of this preparation was mainly directed at the protein components at the cell surface and in the extracellular matrix. That this inhibitory effect on mesodermal erythropoiesis is indeed largely due to the presence of the contaminating enzymes in the collagenase preparation is clearly demonstrated by the finding that whereas the presence of 0,2% 'impure' collagenase (Sigma C-6885) resulted in a total suppression of haemoglobin synthesis by the stage 4 opaca mesodermal explants, the presence of 0,2% highly purified collagenase (Sigma C-3267) did not suppress the erythropoietic differentiation of the same type of explants (Experiment 5.VI).

This latter observation, that the treatment of the explants with the highly purified collagenase (Sigma C-3267) did not directly affect the erythropoietic differentiation of the isolated stage 4 opaca mesodermal cells themselves, is in accordance with the report of Murphy and Carlson (1978) that the vascular associated (i.e., mesodermal associated) collagenous basal lamina materials are laid down only after the erythro-

poietic differentiation of the mesodermal cells is already well under way (see Section 5.3.2). Since the mesodermal-associated collagen is laid down at such a late stage, it is not unexpected that the removal of collagen (by collagenase treatment) does not appear to affect the erythropoietic differentiation of the isolated mesodermal fragments themselves. Indeed, it is conceivable that the vascular-associated collagenous extracellular matrix serves to stabilize and consolidate the walls of the blood vessels themselves.

On the other hand, the fact that the collagenase component itself can also affect cell differentiation is clearly demonstrated in the finding that the presence of 0,2% highly purified collagenase (Sigma C-3267) caused a significant reduction in the erythropoietic differentiation of the stage 8-9 opaca mesoderm-plus-endodermal explants (Experiment 5.VII). Not only did the highly purified collagenase (Sigma C-3267) cause a significant reduction in the erythropoietic differentiation of these mesoderm-plus-endodermal explants, but it reduced the erythropoietic differentiation of these explants to a level comparable with that of the isolated stage 4 opaca mesodermal fragments (i.e., mesoderm cultured in the absence of the endoderm) (Experiment 5.VI). This suggests that the treatment with the highly purified collagenase (Sigma C-3267) may have counteracted the stimulatory influence of the opaca endoderm on mesodermal erythropoiesis. Supportive evidence in favour of this view comes from the fact that when the blastoderm fragments were cultured in the presence of the collagenase, they tended to lose their tissue integrity, and take on the appearance of a mass of loosely aggregated cells. It is conceivable that the dissociation of the cells in these opaca mesoderm-plus-endodermal explants may have prevented the occurrence of the necessary interactions between the endodermal and mesodermal layers, preventing the opaca endoderm from exerting its 'permissive' effect on the mesoderm. This view is further strengthened by the fact that the extent of erythropoiesis of the isolated opaca mesodermal fragments (i.e., mesoderm in the absence of the endoderm) was not affected by treatment with the highly purified collagenase (Sigma C-3267), although these explants likewise took on the appearance of a mass of loosely aggregated cells.

The question then arises as to whether it is the endodermal cells themselves, or their associated extracellular matrix materials, which

are responsible for the 'permissive' effect of the endoderm on mesodermal erythropoiesis. This question arises in view of the findings of previous authors that certain of the classical embryological inductions are mediated by the extracellular matrix components produced by the inductor tissues themselves: In the case of the induction of primary corneal differentiation by the lens, it has been found that collagen and/or certain glycosaminoglycans can effectively substitute for the lens in stimulating the differentiation of the primary corneal stroma (Meier and Hay, 1974a,b; see also Hay, 1977). Similarly, collagen and/or certain proteoglycans can effectively substitute for the notochord in stimulating somitic chondrogenesis (Kosher *et al*, 1973; Kosher and Church, 1975; Lash and Vasan, 1977, 1978). It would be of interest to know whether collagen and/or other extracellular matrix components can effectively substitute for the opaca endoderm in stimulating mesodermal erythropoiesis.

The loss of tissue integrity in consequence of collagenase treatment was not apparent when the explants composed of all three germinal layers of the stage 8-9 ACV were cultured in the presence of the highly purified collagenase (Sigma C-3267). Coupled with this observation, it was found that the treatment of these latter explants with this highly purified collagenase had no effect on their erythropoietic differentiation. It is suggested by the present author that the use of these intact tissue explants may have hampered the penetration of the collagenase throughout the tissue. Since the integrity of the tissue was not obviously undermined, it is conceivable that the appropriate interactions between the germinal layers were able to take place.

CHAPTER SIX

AN INVESTIGATION OF THE EFFECT OF FLATTENING EXPLANTS BY PHYSICAL MEANS ON MESODERMAL ERYTHROPOIESIS: COVERSLIP CULTURE OF BLASTODERM FRAGMENTS IN SMALL DROPS OF MEDIUM

6.1 Introduction

A well known observation from the *in vitro* cultivation of tissue explants is that cells which spread out and become highly flattened in culture do not differentiate, or may dedifferentiate (see Grobstein, 1959, 1965; Holtzer *et al*, 1960; Hilfer, 1962; Whittaker, 1963; Cahn, 1968). For example, it is commonly found that cells which move outward from the explant into the medium, either by individual migration or through the spreading of cohesive sheets, show few overt differentiative characteristics. On the other hand, those parts of the explant in which the integrity of the tissue is preserved and where all essential components are present in reasonably normal relations, are usually found to undergo essentially normal patterns of differentiation (see Grobstein, 1959, 1965). As suggested by Grobstein (1965), " . . . general culture environments which preserve the integrity of relatively complex populations of cells have favoured differentiation, whereas environments and factors which disperse and simplify the relations within such populations have tended to inhibit differentiation." (Grobstein, 1965 page 470).

Such an observation has also been made in the present thesis, in which it has been found that the tendency of the explants to spread out and become flattened over the surface of the coverslips may be coupled with the suppression of mesodermal erythropoiesis (see Chapter Four). Indeed, the observed inhibitory effect of the ectoderm on mesodermal erythropoiesis is often found to be associated with the tendency of the ectoderm-plus-mesodermal explants, in the absence of the endoderm, to spread out and become flattened (see Chapter Four).

In the present chapter attention has been focused upon this correlation between the flattening of the tissue and the suppression of mesodermal erythropoiesis. The coverslip culture technique has been used, and the explants have been flattened either by (a) surface tension

forces, or (b) by the pressure exerted by the application of an inert, permeable membrane to the surface of the explants. As described in detail below, it is found that if the explants remain in a flattened state throughout the culturing period, then haemoglobin synthesis is suppressed. If, on the other hand, the explants become rounded again during the culturing period, then the erythropoietic differentiation of the explants proceeds apparently normally.

6.2 Materials and Methods

0,5mm x 0,5mm explants composed of the stage 8-9 opaca mesoderm-plus-endoderm were cultured in small drops of medium on the surface of coverslips below cavity slides, as described in detail in Section 4.2. However, in the present experiments the tissue fragments were flattened by one of the following means:

1. Medium was removed from the drop so that the tissue was flattened by surface tension forces, or
2. A 1mm x 1mm or 2mm x 2mm square of dialysis membrane or teflon 'CO_2' membrane (Radiometer, Copenhagen) was applied to the surface of the tissue, below which the tissue was gently flattened. Dialysis membranes and teflon 'CO_2' membranes were chosen due to their permeability to gases and small nutrient molecules, thus allowing for the free exchange (by diffusion) of the respiratory gases and small nutrient molecules.

6.3 Experiments and Results

The tissue chosen for use in this study of the effect of physical flattening on mesodermal erythropoiesis was the stage 8-9 opaca mesoderm-plus-endoderm. This is tissue in which the blood-islands have already clumped against the opaca endoderm and are, during normal development, in the process of their erythropoietic differentiation (see Chapter Two). Thus, any effect of flattening on mesodermal erythropoiesis would interfere with the overt differentiation of these blood-island cells.

In this study the tissue explants have been flattened either by surface tension forces (Experiment 6.1), or by the application of an inert, permeable membrane to the surface of the explants (Experiments

6.II and 6.III). A comparison has been made of the extent of erythropoiesis of the explants when:

1. subjected to physical flattening throughout the entire culturing period;
2. subjected to physical flattening for only part of the culturing period; or
3. not subjected to physical flattening.

The details of the experiments performed and results obtained are presented below, and summarized in Table 6.1.

6 3.1 Culture experiments

Experiment 6.I (meso + endo (AOV, st 8-9) flattened (by surface tension forces)

In this experiment the effect of flattening the explants by surface tension forces was investigated. 0,5mm x 0,5mm fragments of the stage 8-9 opaca mesoderm-plus-endoderm were isolated from the posteriolateral AOV (Fig. 6.1) and transferred into a drop of culture medium on the surface of the coverslip.

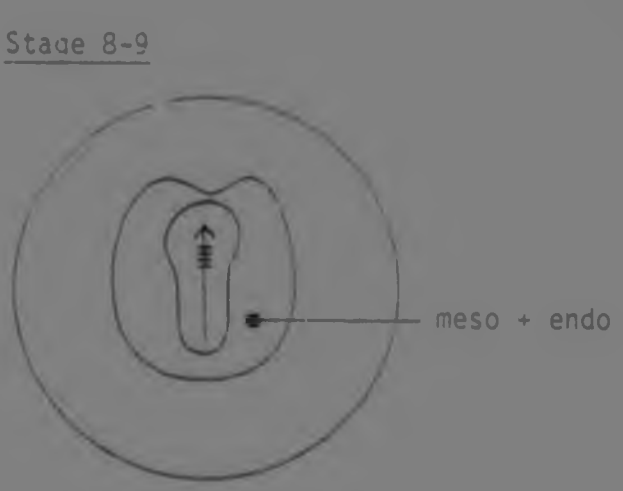


Figure 6.1

Diagram illustrating the area from where mesoderm-plus-endodermal fragments were isolated from the AOV of stage 8-9 blastoderms.

In order to flatten the tissue, excess culture medium was pipetted from the drops so that the surface of the explants just 'dimpled' the surface of the culture medium. In approximately 40 of these cultures

(13 cases out of 31) the tissue became flattened during the culturing period. However, in 60% of the cultures (18 cases out of 31) the drop of culture medium tended to become rounded again during the culturing period, and the tissue was likewise found to have become rounded. A comparison was made of the extent of erythropoiesis of the explants cultured in the above two situations with that of the same type of explants when cultured in medium without being flattened.

In the 13 cases out of 31, in which the tissue remained flattened throughout the culturing period,

- 53,8% of the explants (7 cases out of 13) stained the blue colour of the counterstain only (i.e., the staining for haemoglobin was negative);

-+ 30,8% of the explants (4 cases out of 13) exhibited traces of pale yellow staining (i.e., uncertain haemoglobin staining);

+ 15,4% of the explants (2 cases out of 13) exhibited pale brown (i.e., pale haemoglobin) staining in a large number of cells.

These results are significantly lower than that of the same type of explants (i.e., stage 8-9 opaca mesoderm-plus-endoderm) when cultured in medium without being flattened by surface tension forces. As tabulated hereunder, when the stage 8-9 opaca mesoderm-plus-endodermal explants were cultured in medium without being subjected to physical flattening,

+ 3,8% of the explants (1 case out of 26) exhibited pale brown (i.e., pale haemoglobin) staining in a large number of cells;

++ 19,2% of the explants (5 cases out of 26) exhibited more intense haemoglobin staining in a large number of cells;

+++ 76,9% of the explants (20 cases out of 26) exhibited very intense haemoglobin staining in a large number of cells.

Thus, it is found that when the stage 8-9 opaca mesoderm-plus-endodermal fragments are physically flattened by surface tension forces throughout the culturing period, there is a highly significant suppression in their erythropoietic differentiation (p = much less than 0,001).

On the other hand, in the 18 cases out of 31 in which the drop of culture medium tended to become rounded again during the culturing period, with the resultant rounding up of the explants, the degree of erythropoiesis was found to be very comparable with that of the same

type of explants which were not subjected to physical flattening (p = greater than 0,5). As tabulated hereunder, when the explants became rounded again during the culturing period,

++ 22,2 of the explants (4 cases out of 18) exhibited fairly intense haemoglobin staining in a large number of cells;

+++ 77,8 of the explants (14 cases out of 18) exhibited very intense haemoglobin staining in a large number of cells.

The results of this experiment thus demonstrate that

1. the flattening of the blastoderm fragments (by surface tension forces) results in the suppression of their erythropoietic differentiation and

2. after a period of being flattened by the surface tension of the drop, the explants which become rounded again are able to proceed with their normal differentiation.

In view of the fact that the siliconized[1] surfaces of the coverslips tended to render the drops of culture medium round (as occurred in 60 of the present cases), the experiment was repeated using coverslips which had not been siliconized. The effect of using non-siliconized coverslips was that the drop of culture medium tended to spread over the surface of the glass, flattening the tissue explants as it did so. This treatment resulted in a highly significant suppression in the erythropoietic differentiation of the explants[2] (p = much less than 0,001), as tabulated hereunder,

- 82,4 of the explants (14 cases out of 17) stained the blue colour of the counterstain only (i.e., the staining for haemoglobin was negative);

-+ 17,6 of the explants (3 cases out of 17) exhibited traces of pale yellow staining (i.e., uncertain haemoglobin staining).

In the next pair of experiments in which a less severe method of flattening the explants was used, a similar correlation between the flattening of the explants and the suppression of mesodermal erythro-

---

1. The coverslips employed in the present thesis were routinely siliconized in order to render the drops of culture medium rounded, i.e., whereas the drops of culture medium tend to spread out and 'wet' the surfaces of untreated (unsiliconized) coverslips, they remain rounded and do not spread out on siliconized coverslips.

2. It was for this reason that it was necessary to routinely use siliconized coverslips, rather than untreated coverslips, in all the other coverslip culture experiments of the present thesis.

poiesis was observed (Experiments 6.II and 6.III).

Experiment 6.II (meso + endo (AOV, st 8-9) flattened below a (1mm x 1mm piece of an inert, permeable (membrane

0,5mm x 0,5mm fragments of the stage 8-9 opaca mesoderm-plus-endoderm were isolated from the posterio-lateral AOV (Fig. 6.2) and transferred into a drop of cultur medium on the surface of the coverslip[1].

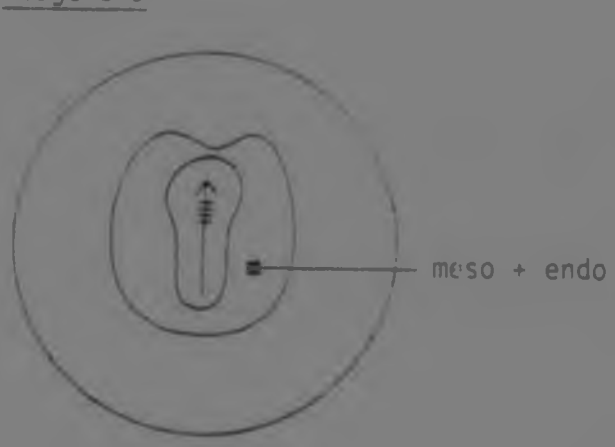


Figure 6.2

Diagram illustrating the area from where mesoderm-plus-endodermal fragments were isolated from the AOV of stage 8-9 blastoderms.

The tissue fragments were then gently flattened by the application to their surfaces of a 1mm x 1mm square of an inert, permeable membrane (dialysis membrane or teflon 'CO_2' membrane). As the piece of membrane was gently pushed down upon the surface of the explants, one could clearly see the tissue expand and spread out beneath it. In most cases the tissue was then cultured for 72 hours with the membrane positioned as above. In some cases, however, the tissue was initially flattened and allowed to expand in the above manner for 3-5 minutes only, whereafter the membrane was removed and the explants cultured for 72 hours without being subjected to further physical flattening. A comparison was made between the extent of erythro-

1. As usual, the coverslips were siliconized.

poiesis of the explants cultured under the above two conditions with that of the same type of explants when cultured in medium without being flattened.

In the cases in which the tissue fragments were initially flattened and allowed to expand under the square of membrane for 3-5 minutes, whereafter the membrane was removed and the explants cultured for 72 hours without being further flattened, the extent of erythropoiesis was very comparable with that of the same type of explants which were cultured without being subjected to physical flattening. As tabulated hereunder, of the explants flattened for 3-5 minutes only,

\+ 10% of the explants (1 case out of 10) exhibited pale brown (i.e., pale haemoglobin) staining in a large number of cells;

++ 20% of the explants (2 cases out of 10) exhibited more intense haemoglobin staining in a large number of cells;

+++ 70% of the explants (7 cases out of 10) exhibited very intense haemoglobin staining in a large number of cells.

These results are not significantly different from that of the same type of explants which were cultured without being physically flattened, as tabulated hereunder (p = greater than 0,5).

\+ 3,8% of the explants (1 case out of 26) exhibited pale brown (i.e., pale haemoglobin) staining in a large number of cells;

++ 19,2% of the explants (5 cases out of 26) exhibited more intense haemoglobin staining in a large number of cells;

+++ 76,9% of the explants (20 cases out of 26) exhibited very intense haemoglobin staining in a large number of cells.

Thus, the brief flattening of the explants by the application and subsequent removal of the squares of membrane did not appear to affect the subsequent erythropoietic differentiation of the explants.

When, on the other hand, the explants were flattened and cultured for 72 hours beneath the squares of inert, permeable membrane, there was found to be a significant suppression in their erythropoietic differentiation. As tabulated hereunder, when the tissue was cultured for 72 hours below 1mm x 1mm squares of dialysis membrane,

-+ 10,5% of the explants (2 cases out of 19) exhibited traces of pale yellow staining (i.e., uncertain haemoglobin staining);

+ 10,5% of the explants (2 cases out of 19) exhibited pale brown (i.e., pale haemoglobin) staining in a large number of cells;

++ 42,1% of the explants (8 cases out of 19) exhibited more intense haemoglobin staining in a large number of cells;

+++ 36,8% of the explants (7 cases out of 19) exhibited very intense haemoglobin staining in a large number of cells.

These results are significantly lower than that of the same type of explants when cultured without being physically flattened (p = less than 0,005). Likewise, when the tissue was flattened below 1mm x 1mm squares of teflon 'CO_2' membrane,

+ 52,9% of the explants (9 cases out of 17) exhibited pale brown (i.e., pale haemoglobin) staining in a large number of cells;

++ 23,5% of the explants (4 cases out of 17) exhibited more intense haemoglobin staining in a large number of cells;

+++ 23,5% of the explants (4 cases out of 17) exhibited very intense haemoglobin staining in a large number of cells.

Again, these results are significantly lower than that of the same type of explants which were not subjected to physical flattening (p = less than 0,001).

A point of relevance noted in these explants which were cultured for 72 hours below the 1mm x 1mm squares of membrane was that the explants in categories -+ and + tended to remain flattened under the squares of membrane throughout the culturing period (Fig. 6.3, left hand micrograph), whereas the explants in categories ++ and +++ were seen to have become more rounded during the culturing period, and were either only partly under (Fig. 6.3, middle micrograph) or completely outside of the membrane (Fig. 6.3, right hand micrograph) at the end of the culturing period. This trend is clearly demonstrated in Fig. 6.4, in which the results of the present experiment have been presented in the form of histograms. A separate histogram has been plotted for each category of haemoglobin staining intensity. Each histogram shows the number of explants in a particular haemoglobin staining category which either remained under the membrane throughout the culturing period, or were partly outside of, or totally free of, the membrane at



Figure 6.3

Photomicrographs showing the appearance, after 72 hours of culture, of the stage 8-9 opaca mesoderm-plus-endodermal explants which had been flattened by the application of a 1mm x 1mm square of dialysis membrane or teflon 'CO_2' membrane to their surfaces. The left hand micrograph shows the appearance of an explant which has remained flattened below the membrane throughout the entire culturing period. The middle micrograph shows an example in which only part of the explant has remained under the membrane, while part is now outside of the membrane. The right hand micrograph shows an explant which is almost completely outside of the membrane. Whereas the explant in the left hand micrograph is seen to have remained flattened throughout the culturing period, the explants in the middle and right hand micrographs are seen (by focusing the microscope at different planes) to have become more rounded during the culturing period.

x 133

the end of the culturing period. As shown in Fig. 6.4, only 3 out of 19 explants (i.e., 15,8 of the cases) were found to have remained flattened beneath the 1mm x 1mm membranes throughout the culturing period. A larger number of explants (5 cases out of 19, i.e., 26,3%) was found to be only partly below the membranes, with the majority of explants (11 cases out of 19, i.e., 57,9%) being rounded and totally free of the membrane at the end of the culturing period. In view of this fact that most of the explants failed to remain flattened under the membrane throu nout the culturing period, the next experiment was carried out in which larger pieces of dialysis membrane (2mm x 2mm) were used.

(Text continues on page 283).

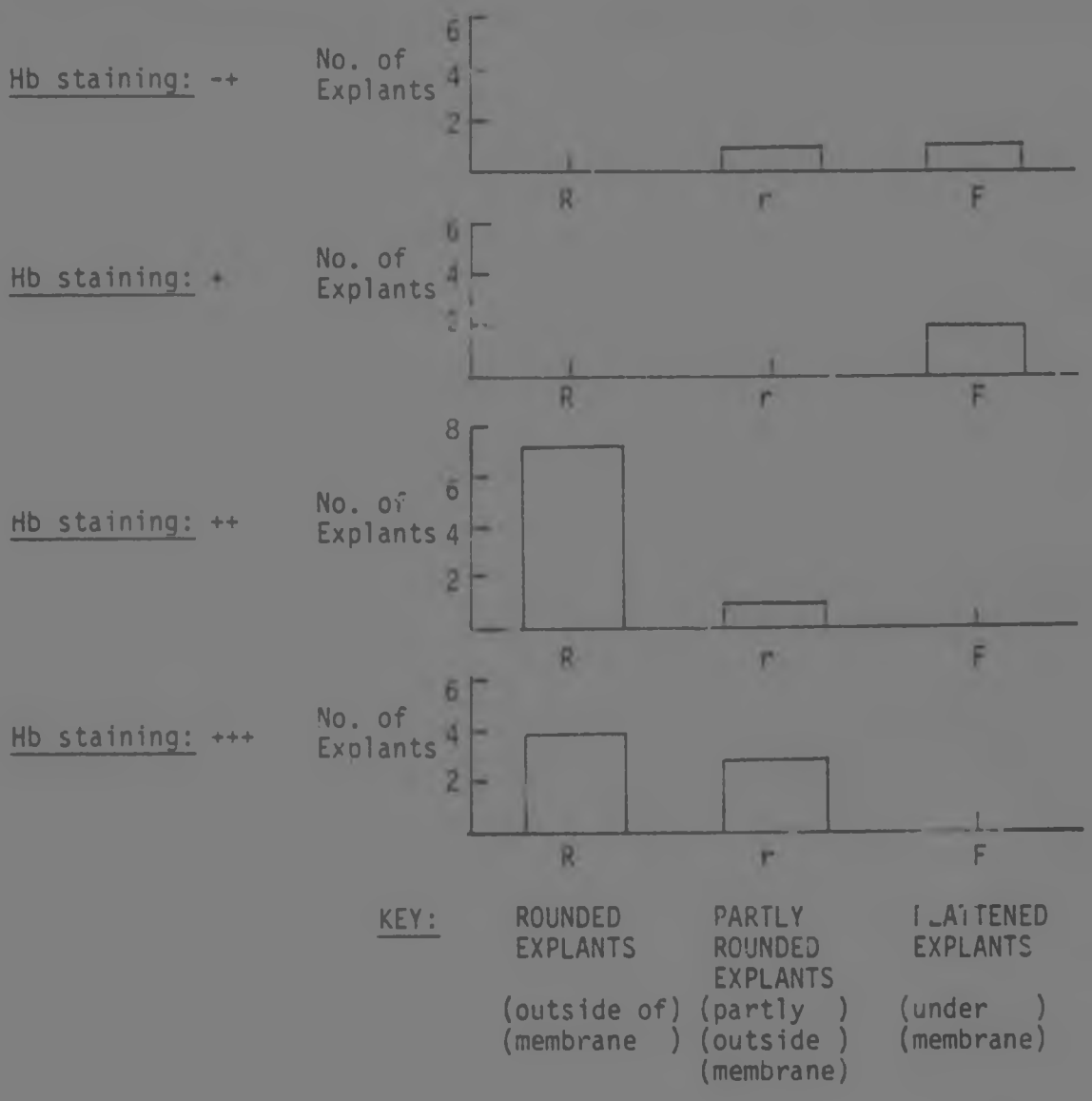


Figure 6.4

Histograms of the results of Experiment 6.II, in which stage 8-9 opaca mesoderm-plus-endodermal fragments were flattened below 1mm x 1mm squares of dialysis membrane and cultured for 72 hours. The results from each category of haemoglobin staining intensity have been plotted in a separate histogram. Each histogram shows the number of explants in a particular haemoglobin category which either remained flattened below the membrane, or which became more rounded and was found to be partially outside of or totally outside of the membrane at the end of the culturing period. The results indicate the tendency for the explants in the 'positive' categories (i.e., categories ++ and +++) to be partially or totally outside of the membrane, and to be rounded. On the other hand, there appears to be a tendency for the small sample of explants in the 'less positive' categories (categories -+ and +) to have remained flattened beneath the membrane.

Experiment 6.III (meso + endo (AOV, st 8-9) flattened below a 2mm x 2mm piece of an inert, permeable membrane)

0,5mm x 0,5mm fragments of the stage 8-9 opaca mesoderm-plus-endoderm were isolated from the posterio-lateral AOV (see Fig. 6.2) and transferred into a drop of culture medium on the surface of the coverslip. The tissue fragments were then gently flattened by the application to their surfaces of a 2mm x 2mm square of dialysis membrane. As the piece of membrane was gently pushed down upon the surface of the explants, one could clearly see the tissue expand and spread out beneath it. The use of these larger pieces of dialysis membrane resulted in a larger proportion of the explants remaining beneath the membrane throughout the culturing period (46,6% when the 2mm x 2mm squares of membrane were used, as compared to 15,8% when the 1mm x 1mm squares of membrane were used) (compare histograms in Figs. 6.4 and 6.5). Furthermore, when the extent of erythropoiesis of the present explants was compared with that of the explants cultured below 1mm x 1mm squares of dialysis membrane, it was found that the use of the larger squares of dialysis membrane (2mm x 2mm) was accompanied by a further significant reduction in the erythropoietic differentiation of the explants ($p = 0,01$), as tabulated hereunder.

- 20% of the explants (3 cases out of 15) stained the blue colour of the counterstain only (i.e., the staining for haemoglobin was negative);
-+ 33,3% of the explants (5 cases out of 15) exhibited traces of pale yellow staining (i.e., uncertain haemoglobin staining);
+ 13,3% of the explants (2 cases out of 15) exhibited pale brown (i.e., pale haemoglobin) staining in a large number of cells;
++ 20% of the explants (3 cases out of 15) exhibited more intense haemoglobin staining in a large number of cells;
+++ 13,3% of the explants (2 cases out of 15) exhibited very intense haemoglobin staining in a large number of cells.

Again, it was found that there was a correlation between the tendency of the explants to remain flattened beneath the dialysis membrane and the suppression of haemoglobin synthesis. This trend is clearly seen in Fig. 6.5, in which the results of the present experiment have been plotted in the form of histograms showing the number of explants

(Text continues on page 286).

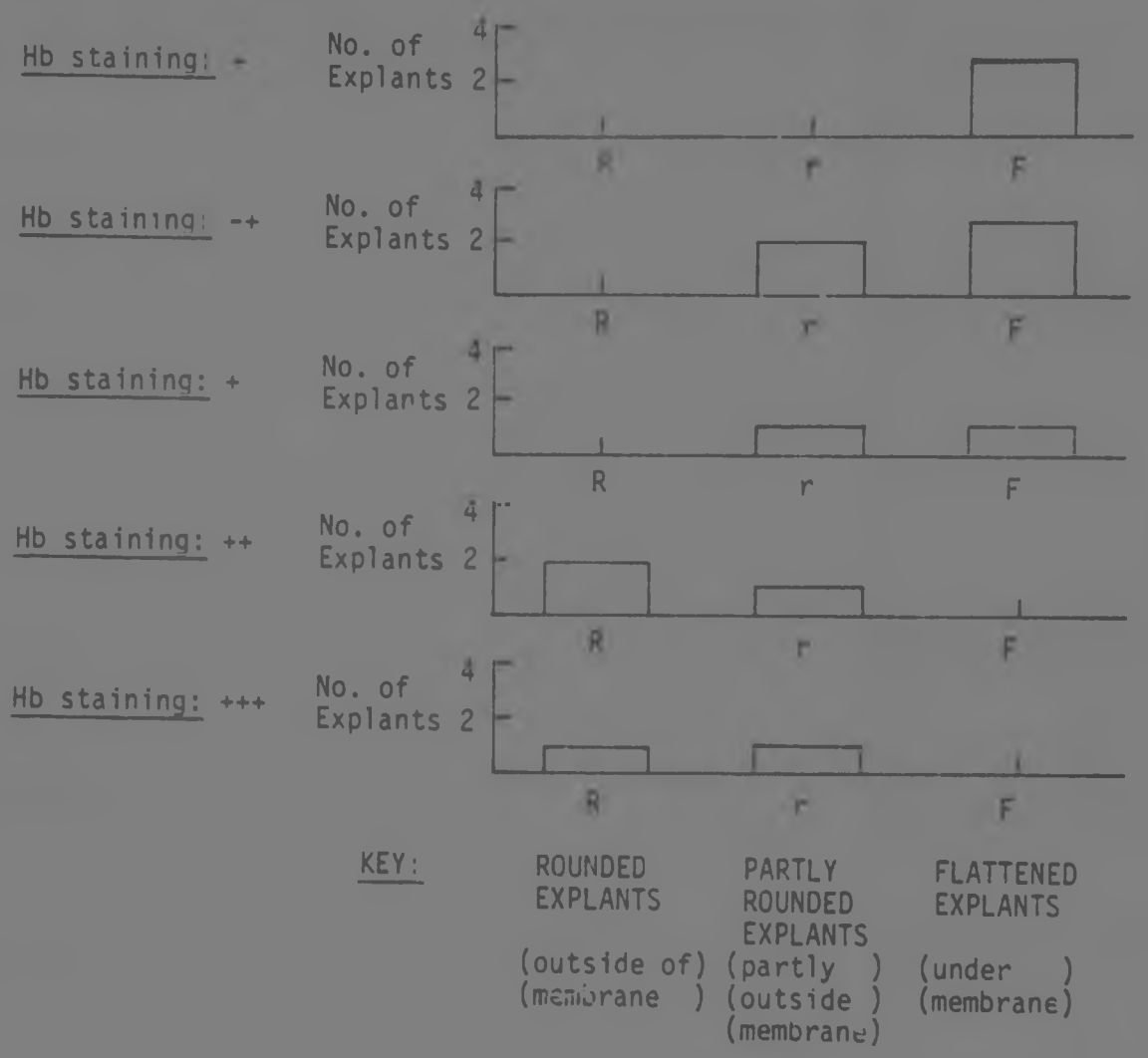


Figure 6.5

Histograms of the results of Experiment 6.III, in which stage 8-9 opaca mesoderm-plus-endodermal fragments were flattened below 2mm x 2mm squares of dialysis membrane and cultured for 72 hours. The results from each category of haemoglobin staining intensity have been plotted in a separate histogram. Each histogram shows the number of explants in a particular haemoglobin category which remained flattened beneath the membrane, or which became more rounded and was found to be partially outside of, or totally outside of, the membrane at the end of the culturing period. The use of the larger squares of dialysis membrane (2mm x 2mm) resulted in a larger proportion of the explants remaining flattened beneath the membrane throughout the culturing period (cf. Fig. 6.4). This was accompanied by a suppression in the erythropoietic differentiation of these explants.

Table 6.I Table summarizing the results of the study to investigate the effect of flattening the explants by physical means on mesodermal erythropoiesis.

Experiments	Types of Explants	Method of Flattening	Total No. of Cases	Categories of Haemoglobin Staining (See key, Fig. 3.1 page 171) No. of Explants					% of Total No.				
				–	-+	+	++	+++	–	-+	+	++	+++
Expt. 6.I	meso + endo (AOV, st 8-9)	surface tension	13	7	4	2	0	0	53,8	30,8	15,4	0,0	0,0
		briefly, surface tension	18	0	0	0	4	14	0,0	0,0	0,0	22,2	77,8
		surface tension, coverslips not siliconized	17	14	3	0	0	0	82,4	17,6	0,0	0,0	0,0
		not flattened	26	0	0	1	5	20	0,0	0,0	3,8	19,2	76,9
Expt. 6.II	meso + endo (AOV, st 8-9)	1mm² dialysis membrane	19	0	2	2	8	7	0,0	10,5	10,5	42,1	36,8
		briefly, 1mm² dialysis membrane	10	0	0	1	2	7	0,0	0,0	10,0	20,0	70,0
		1mm² teflon membrane	17	0	0	9	4	4	0,0	0,0	52,9	23,5	23,5
		not flattened	26	0	0	1	5	20	0,0	0,0	3,8	19,2	76,9
Expt. 6.III	meso + endo (AOV, st 8-9)	4mm² dialysis membrane	15	3	5	2	3	2	20,0	33,3	13,3	20,0	13,3
		not flattened	26	0	0	1	5	20	0,0	0,0	3,8	19,2	76,9

which remained under the membrane, or were partially or totally outside of the membrane at the end of the culturing period. Each category of haemoglobin staining intensity has been plotted in a separate histogram. It was found (by focusing the microscope at different planes) that the explants which were outside of the membrane at the end of the culturing period had become more rounded and had tended to differentiate erythropoietically during the culturing period, whereas those explants which remained under the membrane during the culturing period tended to have remained flattened and did not tend to differentiate erythropoietically during the culturing period.

Summarizing the results of the present study in which the effect of physical flattening the explants on mesodermal erythropoiesis was investigated, it was consistently found that the erythropoietic differentiation of the stage 8-9 opaca mesoderm-plus-endodermal explants may be suppressed if the explants are flattened by physical means (i.e., by surface tension forces, or beneath squares of an inert, permeable membrane). It was found that if the explants remained in a flattened state throughout the culturing period, then haemoglobin synthesis was suppressed. If, on the other hand, the explants became rounded again during the culturing period, then the erythropoietic differentiation of the explants appeared to proceed apparently normally.

6.4 Discussion

The finding from the present study that the flattening of the blastoderm explants is accompanied by a suppression in their erythropoietic differentiation supports previous observations made both in the present thesis (Chapter Four) and in the work of other authors (see, for example, Grobstein, 1959, 1965) that the spreading and flattening of explants in culture may be coupled with the suppression of differentiation.

Of particular relevance to the present observations is the work of Zwilling (1960) on the effect of tissue spreading on cell differentiation. This author found that whereas 0,2mm - 0,3mm diameter reaggregates of the DPS (stage 4) area pellucida tended to spread out rapidly in culture and undergo no neural differentiation, similar reaggregates from older blastoderms (head-fold to 6 somites; stages 6 to 8+) remained cohesive and

massive in culture and produced, among other things, considerable neural tissue. Moreover, when similar reaggregates from these older blastoderms were surgically inserted into a somewhat larger piece of DPS reaggregate, and the combined tissue then grown in culture, 19% failed to express their morphogenetic properties. Zwilling (1960) interprets this result as being due to the altered spreading behaviour of the older blastoderm reaggregates when incorporated into the more rapidly spreading DPS tissue. As described by Zwilling (1960), the older blastoderm reaggregates ". . . were caught in this spreading activity and were forced to spread along with the DPS tissue." (Zwi'ling, 1960 page 30).

Closely related to the aspect of tissue spreading are the concepts of cell population density and tissue mass. It has been repeatedly demonstrated that both the amount of tissue involved in the developing mass, and the size of the fragments into which the original tissue is cut, are factors determining its subsequent morphogenetic behaviour (see Grobstein, 1959, 1965). For example, Zwilling (1960) found that if very small pieces of the older (stages 6 to 8-) blastoderm reaggregates, at or slightly below 0,1mm in diameter, were cultured alone or incorporated into larger pieces of DPS reaggregates, a very high percentage of them (77-89) failed to express a neural differentiation. Indeed, a number of studies carried out by different authors on a variety of tissue types, from sponge to chick and mouse, have consistently shown that the size 0,1mm to 0,2mm diameter is the critical threshold value (or liminal value) of tissue size below which differentiation is suppressed (see Grobstein and Zwilling, 1953).

A relevant study pertaining to both the aspects of explant size and cell population density, in relation to cell differentiation, comes from Grobstein and Zwilling (1953). These authors showed that whereas a standard piece (0,25mm x 0,25mm) of the stage 4 (DPS) chick blastoderm, from the AP just lateral to hensen's node, consistently produced neural tissue when placed on the chick chorioallantoic membrane after 5 days in tissue culture, a piece one-sixteenth the size, treated similarly, never produced nervous tissue although it remained viable. Sixteen such pieces placed in culture in a close cluster, so as to refuse quickly into a single mass, produced nervous tissue on reimplantation in about 30% of the cases. The same number of pieces placed in

a dispersed cluster, so as to spread out more and form a larger but thinner mass of tissue, never produced nervous tissue. This latter situation demonstrates very clearly the importance for both a liminal explant size and liminal cell population density for cell differentiation.

The question then arises as to why explant size, cell population density, and the spreading and flattening of tissues in culture should be causally linked to cell differentiation.

Evidence has been accumulating in recent years which indicates that when cells become flattened and/or spread out in culture they may follow different metabolic pathways compared to when they are rounded and/or clumped. One line of evidence in support of this comes from the cell culture work of Folkman and Moscona (1978) who showed a clear-cut relationship between the extent of cell flattening (i.e., cell shape) and DNA synthesis. In this study, Folkman and Moscona (1978) precisely varied cell shape by a simple modification of substratum adhesiveness and demonstrated, using ^{3}H-labelled thymidine incorporation as an indicator of DNA synthesis, that the flatter the cells the greater the synthesis of DNA. Indeed, as the cells were brought from an extremely flat shape toward a spheroidal conformation, fewer and fewer cells incorporated ^{3}H-thymidine (i.e., the inhibition of DNA synthesis was directly proportional to cell height). It should be noted that in this work of Folkman and Moscona (1978), the authors did not investigate the differential ability of the cells to differentiate when rendered flat or spheroidal; indeed, the cells were physically dissociated from each other. However, it is conceivable that further studies along similar lines, using intact tissues or cell sheets (rather than individual cells), and focused upon a variety of cellular processes which may be associated with cell differentiation (e.g , differential RNA synthesis, protein synthesis, membrane permeability, etc.) may reveal relevant correlations between the flattening and/or spreading of cells *in vitro* and the cellular processes pertaining to overt differentiation.

It should be emphasized, however, that care is in order when extrapolating the *in vitro* situation to the *in vivo* situation. Indeed, in some *in vivo* situations the spreading and flattening of cells may

itself be the expression of an overt differentiation. Examples of this in the yolk sac of the chick blastoderm are the developing vessel endothelia and the developing coelomic linings, which are both flattened sheets of cells (see Chapter Two).

From another perspective, Foster *et al* (1956), in an *in vitro* study of the effect of melanocyte population density on melanin synthesis, drew attention to a possible metabolic basis to explain the relationship between cell population density and cell differentiation. Using frozen sections of tyrosine-active mouse skin in crowded or uncrowded conditions, these workers consistently found that more melanin per hair bulb per weight of tissue was formed in dense than in sparse melanocyte populations. Furthermore, it was found that albino or yellow skin incubated in the presence of this tyrosine-active tissue likewise underwent differential increased darkening. From these observations, Foster *et al* (1956) proposed that the tyrosine-active tissue was producing one or more diffusible substances which were probably intermediates in the sequence from tyrosine to melanin. These authors proposed that their role in increased melanin formation due to crowding may be due to the greater likelihood of the capture of these products in dense compared to sparse populations.

A model invoking a morphogenetic role of diffusible substances produced by the tissue itself was earlier hypothesized by Weiss (1953) to explain the morphogenesis of the cells of the yolk sac blood-islands into either flattened endothelial cells or erythropoietic blood cells. (Refer to Chapter Two for a detailed morphological description of the differentiation of the peripheral cells of the blood-islands into the flattened vessel endothelium and the central cells of the blood-islands into the erythropoietic blood cells). Since the peripheral cells of the blood-islands are seen to give rise to the flattened vessel endothelium, and the central cells of the blood-islands are seen to differentiate into blood cells, one may question whether the peripheral and central cells are inherently different from each other, or whether they are equipotential and that their pathways of differentiation are dependent upon differential environmental factors. This latter possibility was hypothesized by Weiss (1953) who suggested that ". . . the terms 'outer' and 'inner' refer not to properties intrinsic to the component cells but to differentials conferred upon them by virtue of their position

within the group." (Weiss, 1953 pages 208-209). According to Weiss (1953), the blood-island represents a simple equipotential cell cluster. Weiss further assumed that if the cells are engaged in activities producing metabolites, these will be most concentrated in the centre of the clump, with a gradient of activity tapering outwards towards the periphery of the clump. On the other hand, the peripheral cells of the clump may border on cells of some other type (e.g., the yolk sac endoderm or the surrounding mesenchymal cells) or on some other medium (e.g., the components of the extracellular spaces) (refer to Fig. 4.30 page 224). As a result of interactions along their free surface, a certain course of reactions may be activated in these cells at the periphery of the clump. This may entail the release from these cells of other substances or activities which may spread inwards with a gradient from their source. Additional factors of this kind may be the differential accessibility of the peripheral and central cells to essential food constituents, oxygen supply, and so on (Weiss, 1953). The overall result is that a number of gradients of metabolic products or activities will be set up, with high points and low points at either the periphery or centre of the clump. Depending upon the position of the cells in the clump, so they will be subjected to a particular constellation or combination of such factors. Weiss (1953) proposed that the cells react to these factors by a specific differentiation step, there being a given threshold level of particular factors for this reaction. In the central area of the clump, where the concentration of metabolites agrees with a certain requisite threshold intensity, the cells will be switched into the course of differentiation leading to erythropoiesis. At the periphery of the clump, where the concentrations of metabolites and activities are at the other extreme of the composite gradient, the cells will be switched into a course of differentiation leading to endothelium formation. According to this model, if the original cluster of cells were to be rearranged or bi-sected, or if individual cells simply traded places, each cell would then behave according to its actual, rather than its former, position (Weiss, 1953).

It is tempting to apply this model to explain the observations from the present study that the flattening of the blastoderm explants is coupled with a suppression of mesodermal erythropoiesis. In terms of Weiss' model, one may speculate that when the blood-islands are com-

pressed into a flattened layer of tissue, the composite concentration gradients of metabolic products and/or activities between the 'inside' and 'outside' cells would be less steep (or in extreme cases of flattening, totally diminished) and would consequently not provide the required threshold concentration of factors conducive to an erythropoietic differentiation. According to this model, conditions would then favour an endothelial type of differentiation rather than an erythropoietic differentiation.

In view of the explanation provided by Weiss' model, care should be exercised in the present study when interpreting whether the flattening of the blastoderm explants results in a suppression of differentiation or a change in the pathway of differentiation. In terms of Weiss' model, the latter situation would hold (i.e., the blood-island cells would differentiate into endothelial cells rather than erythropoietic cells). If this situation were indeed true, it would be contrary to the common view that the flattening of explants in culture inhibits differentiation (see Grobstein, 1959, 1965). Further work is therefore required to elucidate which of these two possibilities (or even other possibilities) may be operative.

CHAPTER SEVEN

ARE THE BLOOD-ISLAND CELLS IRREVERSIBLY RESTRICTED TO THE FORMATION OF THEIR NORMAL PROGENY LINEAGES (ENDOTHELIUM AND BLOOD CELLS) OR ARE THEY STILL ABLE TO CHANGE THEIR PATHWAYS OF DIFFERENTIATION? AN INVESTIGATION BY THE TRANSPLANTATION OF BLOOD-ISLANDS INTO HOST BLASTODERMS

7.1 Introduction

In the previous discussion (Section 6.4) aspects of the question of what determines the developmental fates of the blood-island cells were discussed. For example, the question was posed as to what determines whether a given blood-island cell will give rise to an endothelial cell or an erythropoietic blood cell. This type of question has been extended in the present chapter (Chapter Seven) to cover a wider focus. The question under investigation is whether the cells of the blood-islands are irreversibly restricted to the formation of either endothelial- or blood cells, or whether they are also capable of giving rise to the other cell types of the yolk sac system, such as the coelomic lining cells.

As described in the histological study of Chapter Two, the primary mesenchymal cells which migrate into the AOV are initially all morphologically identical. The first overt sign of yolk sac vasculogenesis is the clumping together of some of these cells to form the blood-islands. Meanwhile, the remaining cells in the sheet become aligned to form the linings of the extraemoryonic coelom. Thus, from the original sheet of morphologically identical cells, two types of cells become recognizable (i.e., those cells which clump together to form the blood-islands, and those cells which do not clump together, but which spread out to form the coelomic linings).

When the blood-island cells first clump together, they are all morphologically identical with each other (Edmonds, 1964, 1966; Mato *et al*, 1964). However, the outermost blood-island cells soon flatten and form the vessel endothelium whereas the more central blood-island cells subsequently round off and form the intravascular blood cells (see Chapter Two). Thus, from the blood-island cells themselves, two cell types in turn become recognizable.

Likewise, the cells which give rise to the coelomic linings may form either the somatopleuric lining (which lines the ectoderm) or the splanchnopleuric lining (which lines the endoderm and blood vessels, and gives rise to the smooth muscle layer around the blood vessels (see Murphy and Carlson, 1978)). Thus, at each critical period, the mesodermal cells appear to embark upon one of two developmental pathways. This may be represented diagrammatically, as shown in Fig. 7.1.

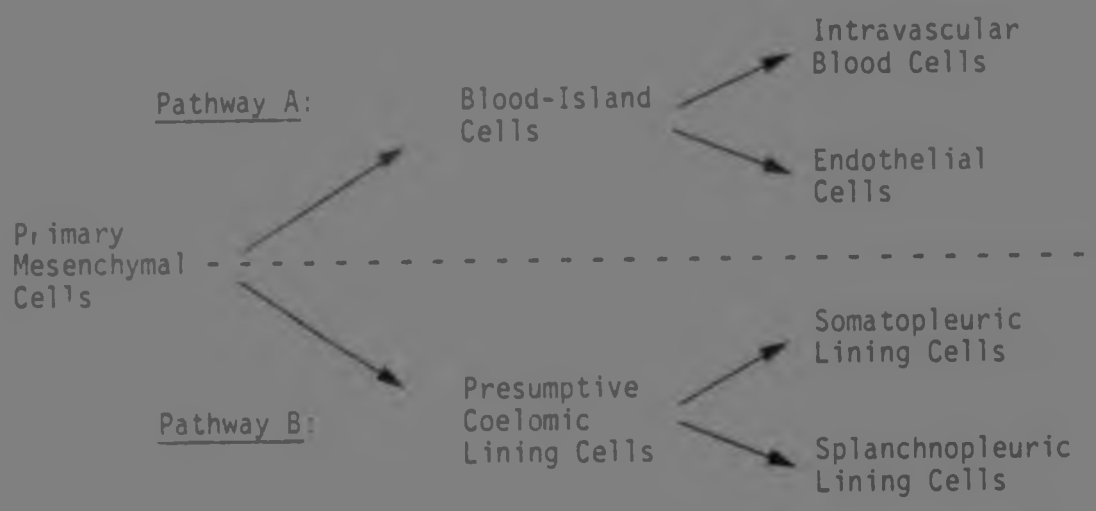


Figure 7.1

Fate map of the determination events which occur during the development of the yolk sac vascular system.

This leads to the question of whether the cells which have embarked upon a given pathway of differentiation are irreversibly committed to forming the progeny cell types of that lineage only, or whether they are still capable of giving rise to the cell types derived from the alternative lineage. For example, are the cells which have already clumped together to form the blood-islands (i.e., the cells which have embarked upon Pathway A in Fig. 7.1) irreversibly restricted to forming only blood cells or endothelial cells (i.e., the progeny cell types of Pathway A), or are they also capable of embarking upon the alternative pathway of differentiation (i.e., Pathway B) leading to the

formation of the coelomic lining cells? The aim of the present study was to investigate this question.

This was done by preparing radioactively labelled blood-islands, transplanting them into the AOV of non-radioactive host blastoderms in culture, and following their developmental fates autoradiographically (see Materials and Methods below). As detailed in Section 7.3 below, it is found that once the blood-island cells have clumped together to form the blood-islands, they appear to be irreversibly committed to forming the progeny cell types of the blood-islands only (i.e., the blood cells and endothelial cells) (see Pathway A of Fig. 7.1).

7.2 Materials and Methods

Radioactively labelled blood-islands for implantation into host blastoderms were prepared by a slight modification of the method employed by Hagopian *et al* (1972). In this procedure, the primary mesenchymal tissue was isolated at a stage prior to the period of blood-island formation (i.e., from stage 3 blastoderms), dissociated into small fragments, and the presumptive blood-island cells subsequently allowed to clump together by swirling the medium at 68rpm on a gyrotary shaker (see details of method in Section 7.2.1. below). As described by Hagopian *et al* (1972), this motion usually keeps the differentiating blood-islands in suspension, allowing the other cell types to attach to the floor of the beaker. The blood-islands were then removed from the medium by means of a micro-syringe, for the subsequent transplantation procedures (see Section 7.2.2 below).

This method of utilizing the presumptive blood-island tissue, and allowing the blood-islands to subsequently form *in vitro*, was employed in view of the difficulty experienced in preliminary attempts to isolate the blood-islands from the blastoderm at a stage after they had already formed (e.g., at stage 5-6). In these early attempts it was found tnat the surrounding mesodermal cells in the sheet tended to adhere to the blood-island clumps, making it difficult to obtain 'uncontaminated' blood-islands (see Fig. 4.30 page 224).

The details of the experimental procedure are outlined below.

7.2.1 Preparation of radioactively labelled blood-islands

All procedures were carried out under sterile conditions, as described in detail in Section 3.2.1.

The 'donor' blastoderms were rendered radioactive by administering 5μCi methyl-^{3}H-thymidine to the unincubated eggs (see details of method in Section 3.2.2.3) and the eggs then incubated at 38°C until they had reached stage 3. The blastoderms were dissected from the yolk under chick embryo saline and removed from their vitelline membranes.

Using sharp stainless-steel insect pins (size 0) (D.F. Tayler and Co., England) mounted on pencil-length glass rods, the hypoblast was removed from the posterior half of the AP, and the underlying epiblast and primary mesenchyme was then excised from the rest of the blastoderm for further use (Fig. 7.2). (Note: This tissue was chosen since a large proportion of the stage 3 epiblastic cells are destined to invaginate through the primitive streak and give rise to the primary mesenchyme - see Chapter Two).



Figure 7.2

Diagram illustrating the dissection procedure in which the posterior half of the stage 3 area pellucida, composed of the epiblast and primary mesenchyme, was excised from the rest of the blastoderm.

Eight such dissected AP halves were pooled in 1ml of liquid culture medium in a 5ml-beaker, the liquid culture medium having been prepared according to the method described in Section 4.2.1.3.

The blastoderm tissue was cut into minute fragments with a pair of fine dissecting scissors, and thereafter gently flushed up and down in a 1mm-diameter pasteur pipette until a fine suspension was obtained (about 10 flushes). Care was taken to avoid frothing the medium during the flushing procedure. The beakers were then sealed with Parafilm (American Can Company, Greenwich) and incubated at 38°C for 8-10 hours on a model G2 Laboratory Gyrotary Rotator (New Brunswick Scientific Company, New Jersey) at a speed of 68rpm.

It was found that the success of isolating 'uncontaminated' blood-islands by the above method depended upon the particular batch of foetal calf serum employed in the culture medium[1]. When one particular batch of foetal calf serum was used, the blood-islands were easily separated from the other cell types owing to the fact that the blood-islands tended to remain in suspension whereas the other cell types tended to become attached to the floor of the beaker. (A similar observation was also described by Hagopian *et al*, 1972). When, however, an alternative batch of foetal calf serum was employed, there was a tendency for all the cell types to remain in suspension. This resulted in the formation in these latter cultures, of blood-islands which were contaminated with the other cell types. Often these contaminating cells tended to form epithelial-like vesicles around the blood-islands.

An obvious difference between the 'uncontaminated' and the 'contaminated' blood-islands was made evident when the blood-islands were subsequently cut into fragments prior to implantation into host blastoderms. It was found that it was possible to make very clean cuts through the 'uncontaminated' blood-islands (as if one were slicing through soft butter). On the other hand, it was not possible to cut through the 'contaminated' blood-islands without some of their cells sticking to the dissecting needles. This made the handling of the 'contaminated' blood-islands relatively difficult.

The formation of these 'contaminated' blood-islands was exploited in the present study. The 'contaminated' blood-islands were likewise transplanted into host blastoderms, and were used as 'controls'

---

1. Hagopian *et al* (1972) also reported the necessity to test for and select suitable batches of foetal calf serum.

to compare the types of developmental pathways embarked upon by the 'contaminated' and 'uncontaminated' blood-islands.

7.2.2 Transplantation of radioactively labelled blood-island fragments into unlabelled host blastoderms

The radioactively labelled blood-islands, prepared as above, were transferred into a petri dish of saline by means of a micro-syringe with a vernier control (Burroughs Wellcome and Company, England; and G.K.N. Shardlow Metrology Ltd., England). Apposing the tips of the insect pins against each other in a shearing motion, the blood-islands were cut into 50-100μm fragments. The blood-island fragments were then transported, by means of the micro-syringe, onto the surface of unlabelled stage 6 host blastoderms for implantation. The host blastoderms were situated on their vitelline membranes within glass rings, on the surface of solid organ culture medium, as described in Sections 3.2.2.1 and 3.2.2.2. The insect pins were used to implant the blood-island fragments into the AOV of the host blastoderms, as detailed in Section 3.2.2.3. At least 20 blood-island fragments were implanted into each host blastoderm, the 'uncontaminated' and 'contaminated' blood-island fragments being kept separate from each other and implanted into 5 separate host blastoderms each. Thereafter, the host blastoderms were cultured overnight at 38°C. The host blastoderms were then fixed and processed, and autoradiographic serial sections were prepared according to the methods described in Section 3.2.2.5.

7.3 Experiments and Results

An examination of the autoradiographic histological serial sections of the host blastoderms into which the 'uncontaminated' blood-island fragments had been implanted (Fig. 7.3) revealed that the labelled cells (i.e., the transplanted blood-island cells) had mainly given rise to dorsal and ventral endothelial cells, and an abundance of intravascular blood cells (Fig. 7.3). These are the progeny cell types of Pathway A (see Fig. 7.1). On the other hand, the somatopleuric and splanchnopleuric coelomic linings tended to be conspicuously unlabelled (Fig. 7.3). These are the progeny cell types of Pathway B (Fig. 7.1) Indeed, of over 2000 labelled cells scored,

(Text continues on page 300).

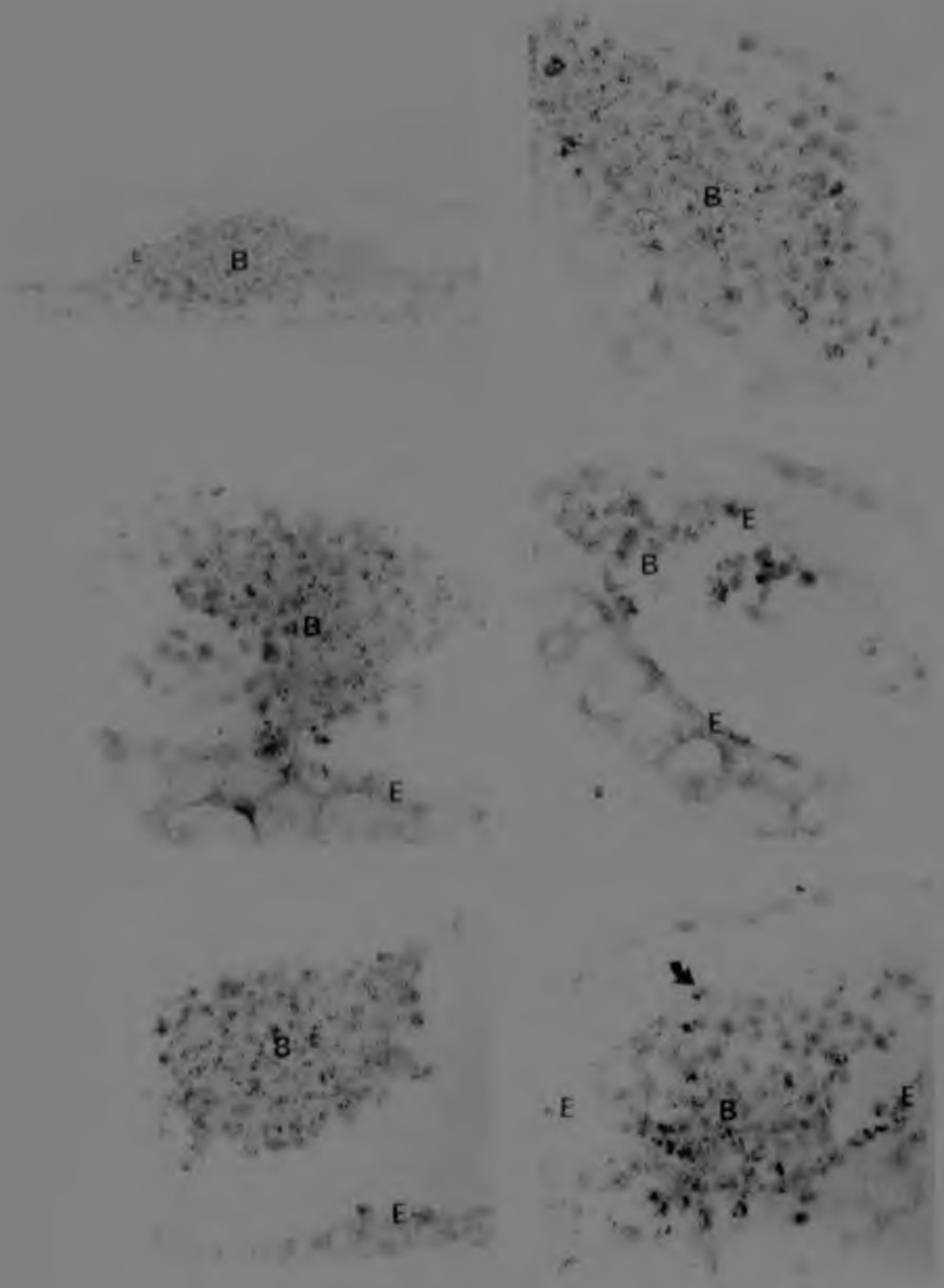


Figure 7.3 (See legend overleaf)

B, blood cells; E, endothelial cells; S, somatopleure; Sp, splanchnopleure; arrow, questionable cell type.

top left micrograph x 356

all others x 670

Figure 7.3 (See photographs on previous page)

Photomicrographs of the autoradiographic histological sections of the AOV of the host blastoderms into which radioactively labelled 'uncontaminated' blood-island fragments had been implanted. The labelled cells (i.e., transplanted cells) are seen to have given rise mainly to the progeny cell types of Pathway A, forming dorsal and ventral endothelial cells (E) and an abundance of intravascular blood cells (B). The somatopleuric (S) and splanchnopleuric (Sp) coelomic lining cells (the progeny cell types of Pathway B) are conspicuously unlabelled. However, in the lower right hand micrograph a situation is shown in which it is difficult to tell with certainty whether one of the labelled cells (arrow) is part of the vessel endothelium or the splanchnopleure.

B, blood cells; E, endothelial cells; S, somatopleure; Sp, splanchnopleure; arrow, questionable cell type.

top left micrograph x 356

all others x 670

less than five percent appeared as if they might have been situated in the coelomic lining (see Fig. 7.3, lower right micrograph). This situation is in direct contrast to that which occurred when the 'contaminated' blood-island fragments were implanted into the host blastoderms, in which case it was found that all the mesodermal cell types (i.e., the endothelial cells, the blood cells, and the somatopleuric and splanchnopleuric lining cells) had become conspicuously labelled (Fig. 7.4). These cells represent the progeny cell types of both Pathway A and Pathway B (see Fig. 7.1). In all cases, the ectoderm and endoderm were found to be unlabelled (Figs. 7.3, 7.4).

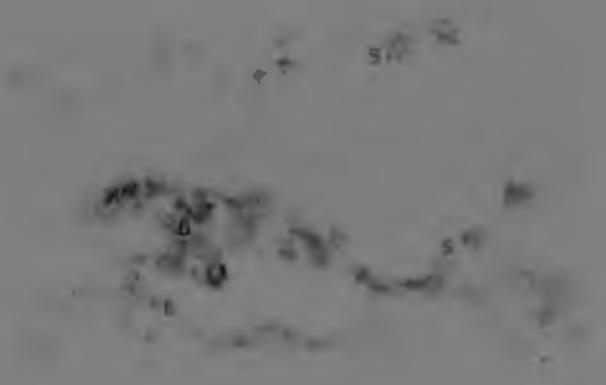


Figure 7.4

Photomicrograph of an autoradiographic histological section of the AOV of a host blastoderm into which radioactively labelled 'contaminated' blood-island fragments had been implanted. All the mesodermal cell-types, i.e., the blood cells (B), the endothelial cells (E), and the somatopleuric and splanchnopleuric coelomic lining cells (S), are seen to have become conspicuously labelled. These labelled cells represent the progeny cell types of both Pathway A and Pathway B.

B, blood cells; E, endothelial cells; S, coelomic lining cells.

x 711

The finding that the 'uncontaminated' blood-island fragments tended to give rise only to endothelial cells and blood cells, whereas the 'contaminated' blood-islands gave rise to all the mesodermal cell types of the AOV, would suggest that the 'uncontaminated' blood-islands were indeed essentially uncontaminated with the other mesodermal cell types. It would appear, furthermore, that at the time of implantation the blood-island cells, which had already clumped together, had already become

restricted to the formation of the progeny cell types of their own lineage, i.e., to blood cells and endothelial cells only (Pathway A of Fig. 7.1).

7.4 Discussion

As indicated in the r[illegible] above the present finding that the cells of the blood-[illegible]nd to give rise only to endothelial cells and blood cells wou[illegible]ndicate that once the blood-island cells have clumped together, they are restricted to forming the progeny cell types of their own lineage only (Pathway A of Fig. 7.1). The fact that a small proportion (less than 5%) of the labelled cells appeared as if they might have been situated in the coelomic lining may be explained by the following possibilities:

1. The original blood-island fragments may have been contaminated with a small number of presumptive coelomic lining cells, or
2. Some of the transplanted blood-island cells may have become artificially displaced as a result of the experimental procedures, or
3. There may have been a small number of cells which were still pluripotent at the time of transplantation.

The question then arises as to when the initial determination of the primary mesenchymal cells into blood-island cells and coelomic lining cells takes place. This question has previously been focused upon by Wilt (1974), who explanted small pieces of the unincubated chick blastoderm *in vitro* and found that only the posterior regions were able subsequently to differentiate erythropoietically. From this, Wilt (1974) concluded that the determination of the presumptive erythropoietic tissue has already occurred by the time the egg is laid (stage 1). This is at the stage when the blastoderm is still only two-layered (i.e., composed of the epiblast and hypoblast), and is many hours before the epiblastic cells will invaginate through the primitive streak to form the primary mesenchymal cells (refer to Chapter Two). It would be of interest to know whether or not at this early stage there are already two sub-populations of presumptive mesodermal cells, i.e., a sub-population of presumptive blood-island cells (which will subsequently embark upon Pathway A of Fig. 7.1) and a sub-population of presumptive coelomic lining cells (which will subsequently

embark upon Pathway B of Fig. 7.1), or whether the two cell-lineages only become discrete from each other at a later stage.

In this regard, studies using ^{3}H-labelled cDNA as a probe for the synthesis of globin mRNA have shown that most of the globin mRNA is only synthesized between the DPS stage (18 hours incubation) and the head-fold stage (24 hours incubation) (Chan *et al*, 1974). These stages correspond to the period during which the primary mesenchymal cells migrate from the AP into the AOV, and when the presumptive blood-island cells then clump together to form the blood-islands (refer to Chapter Two). However, in the study of Chan *et al* (1974), these authors did not indicate the proportion of mesenchymal cells synthesizing globin mRNA during these stages, i.e., it has not been demonstrated whether globin mRNA is synthesized initially by all the primary mesenchymal cells, or is synthesized only by a sub-population of these cells, viz., the presumptive blood-island cells.

However, relevant observations pertaining to this question have been made in other developmental systems. For example, Caplan and Ordahl (1978) report that in the development of the neural crest cells into elements of the sensory and sympathetic nervous system, the progenitor cells contain both cholinergic- and adrenergic specific molecules. As these cells further develop, the synthesis of one or other of these compounds is terminated, coincident with the emergence of specific sensory or sympathetic phenotypes (see Caplan and Ordahl, 1978). Furthermore, rudimentary evidence suggests that during the early development of the limb bud, the progenitor cells to muscle and cartilage may be expressing low levels of synthetic activity for both phenotypes, with the eventual accentuation of one or other of these products when the cells finally differentiate into either muscle or cartilage (Searls, 1965, and Medoff and Zwilling, 1972).

It would be of relevance to determine whether or not a similar type of situation may occur in the primary mesenchymal cells prior to their overt differentiation into blood-island cells on the one hand, and coelomic lining cells on the other.

CHAPTER EIGHT

CONCLUDING DISCUSSION

8.1 Overview and Some Hypotheses

A central feature of embryology concerns the way in which the cells of the embryo become translocated in spatially and temporally defined ways, creating new cellular associations between distinct cell populations (see Trinkaus, 1969, 1976). As highlighted by the pioneering work of Spemann (see Spemann, 1938; Saxén and Toivonen, 1962)), of fundamental importance is the way in which these interactions between dissimilar cell populations alters cell behaviour in developmentally significant ways, which lead to the moulding of the tissue and organ patterns characteristic of the adult organism.

The early development of the yolk sac vascular system of the chick blastoderm has proved to be an ideal model system in which some of these features can be analysed. The early development of this system involves the translocation of the primary mesenchymal cells from the central axis of the blastoderm (the primitive streak) towards a more peripheral location (the AOV), and their subsequent assembly into a simple three-dimensional pattern of blood vessels, blood cells and coelomic spaces (Chapter Two; see also Fig. 8.2 below). During the period of translocation the mesenchymal cells experience changing positional relationships with respect not only to the central axis of the blastoderm, but also to the ectoderm and endoderm of the AP and AOV through which they migrate. In the present study it has been shown that these latter interactions influence the course of differentiation upon which the mesodermal cells will embark, moulding the temporal and spatial patterns of morphogenesis which occur in these regions.

The following discussion focuses upon this morphogenetic patterning of the yolk sac vascular system, and the way in which the associated germinal layers of the different regions of the blastoderm may affect the creation of this pattern.

8.1.1 The morphogenetic patterning of the yolk sac vascular system

In analysing the creation of this three-dimensional pattern of vessels, blood cells, and coelomic spaces from the present SEM and light microscopic study (Chapter Two), a simple flow-diagram illustrating the two main pathways of differentiation of the primary mesenchymal cells has beer drawn up, as shown in Fig. 8.1.

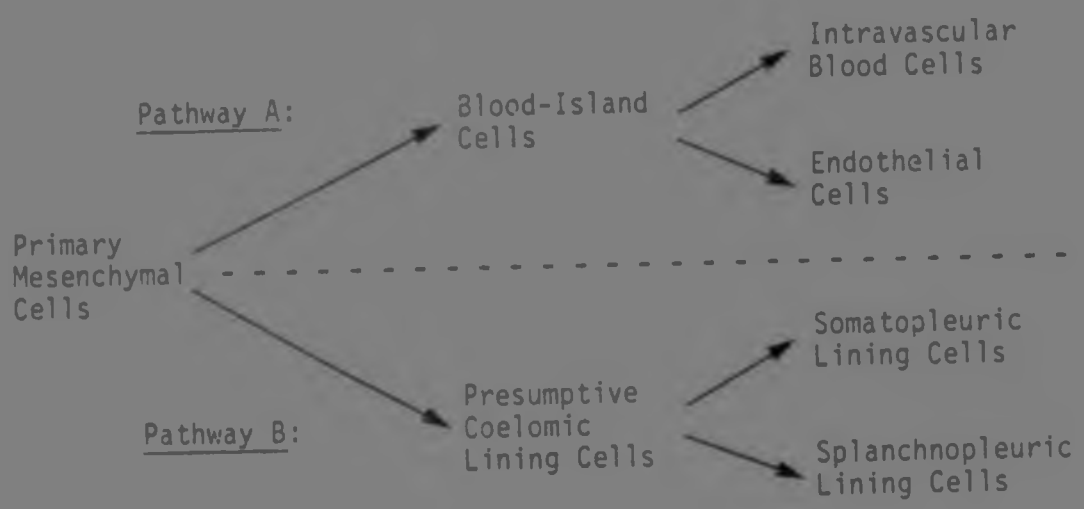


Figure 8.1

Diagram representing the two main cell-lineages (Pathway A and Pathway B) which arise from the primary mesenchymal cells.

As diagrammed in Fig. 8.1 and illustrated in Fig. 8.2, these pathways are:

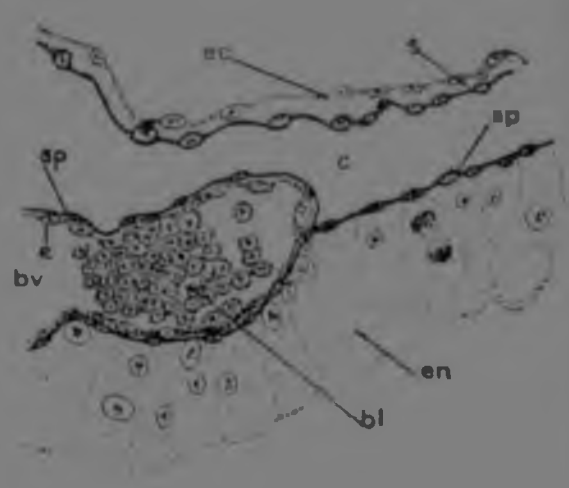
Pathway A : the formation of the blood-islands by the clumping together of some of the primary mesenchymal cells, followed by their subsequent differentiation into blood vessels containing free-floating blood cells (see Chapter Two);

Pathway B : the organization of the remaining primary mesenchymal cells into the sheet-like linings of the extraembryonic coelom, the somatopleuric lining underlying the ectoderm, and the splanchnopleuric lining covering over the underlying blood system and endoderm (see Chapter Two).

It is furthermore found that in the organization of the primary mesenchymal cells into these various components of the pattern (Figs. 8.1 and 8.2), two types of polarization become apparent, a dorso-ventral polarity and a proximo-distal polarity.

1. The Dorso-Ventral Polarity

The clumping together of the prospective blood-island cells in juxtaposition to the underlying opaca endoderm, and the organization of the remaining mesenchymal cells into the sheet-like coelomic linings dorsally, are the visual manifestations of the dorso-ventral polarity (see Chapter Two). This is clearly illustrated in Fig. 8.2, in which the developing blood system is seen to be ventrally compartmentalized together with the endoderm, and separated from the ectoderm dorsally by the large coelomic space.



(After Balinsky, 1981).

Figure 8.2

Drawing illustrating the localization of the differentiating blood-system in a ventral position in juxtaposition to the opaca endoderm, and separated dorsally from the ectoderm by the cell-lined extraembryonic coelom.

bl, blood cells) Pathway A;
bv, blood vessel)
e, endothelium)

c, coelom) Pathway B;
s, somatopleure)
sp, splanchnopleure)

ec, ectoderm; en, endoderm

This close proximity of the developing blood system to the underlying endoderm is in functional accordance with its role in

mobilizing nutrients from the yolk sac to the developing embryo (see Romanoff, 1960; Patten and Carlson, 1974).

In terms of the histological observations of the cellular patterning involved in the morphogenesis of this system (see Chapter Two), the opaca endoderm is seen to be a surface against which the prospective blood-island cells are able to clump together, whereas the overlying ectoderm does not appear to be conducive to the clumping together of these cells, but is rather a surface under which the mesenchymal cells tend to spread out and form flattened sheets of cells. Indeed, when the stage 4 opaca mesoderm is cultured [illegible] in combination with the overlying ectoderm, the explants tend to spread out and become flattened in culture (Experment 4.IX, Chapter Four), and blood-island differentiation is suppressed[1]. On the other hand, when the same type of explants are recombined in culture with the opaca endoderm (Experiment 4.VIII, Chapter Four), the explants no longer exhibit the strong tendency to spread out and flatten, and very obvious erythropoietic blood-islands are formed. These findings support those of Wilt (1965a) and Miura and Wilt (1969). Indeed, Miura and Wilt (1969) reported that when the stage 4 opaca ectoderm-plus-mesodermal explants were cultured in the absence of the endoderm, not only was the frequency of blood-island formation depressed, but the small number of blood-islands formed were themselves so small that half could be seen only by the examination of sectioned material at nigh power. In agreement with Wilt (1965a) and Miura and Wilt (1969), the stage 4 opaca endoderm is thus found to exert a salutary effect on the clumping together of the blood-island cells and their overt erythropoietic differentiation (see Chapter Four).

Besides the above finding that the erythropoietic differentiation of the stage 4 opaca ectoderm-plus-mesodermal explants is suppressed in the absence of the opaca endoderm, a further observation made in the present work is that the overt erythropoietic differentiation of the isolated stage 4 opaca mesoderm, cultured alone, is greater than that of the stage 4 opaca mesoderm cultured in combination

---

1. The question of whether the suppression of differentiation is a consequence of this spreading and/or flattening of the explants is discussed in Chapter Six, and on page 307 below.

with its overlying ectoderm (Experiments 4.IX and 4.X, Chapter Four). This suggests that the opaca ectoderm may exert an inhibitory effect on mesodermal erythropoiesis. This negative effect of the ectoderm on mesodermal erythropoiesis is in perfect developmental harmony with the spatial dorso-ventral polarity exhibited by the components of the developing yolk sac vascular system, the prospective blood-island cells being unable to realize their full developmental potential in the dorsal region of the developing tissue construction. On the other hand, the permissive endoderm, being conducive to clumping and to blood-island differentiation, would ensure the development of the blood system in the ventral part of the pattern.

However, in considering the observed relationship between the spreading and flattening of the ectoderm-plus-mesodermal explants and the suppression of mesodermal erythropoiesis (Experiment 4.IX, Chapter Four), it should be noted that it has long been known that when tissue explants spread out and become flattened in culture, they tend not to differentiate (see Grobstein, 1959, 1965). With this in mind, it has not yet been determined whether the inhibitory effect of the opaca ectoderm on mesodermal erythropoiesis is a consequence of the spreading and/or flattening of the ectoderm-plus-mesodermal explants only, or is due to some other morphogenetic effect of the ectoderm on mesodermal erythropoiesis. It is also not known whether the observed suppression of mesodermal erythropoiesis in these flattened ectoderm-plus-mesodermal explants is due to the suppression of blood-island differentiation only, or due to the suppression of cell differentiation in general. Furthermore, as noted in Chapter Six, the flattening of sheets of tissue may in some instances ever be a manifestation of cell differentiation, as in the case of the developing endothelial linings and coelomic linings of the yolk sac vascular system, both of which develop as flattened sheets of cells (see Fig. 8.2). This therefore elicits the question of whether the spreading and flattening of the ectoderm-plus-mesodermal explants could perhaps have even altered the pathway of differentiation of the prospective erythropoietic cells, causing them to develop into flattened cellular linings (e.g., endothelial cells). The resolution of the above questions awaits further experimentation.

A further aspect concerning the dorso-ventral polarity exhibited by

the mesodermal cells of the yolk sac vascular system is the finding (Experiments in Chapter Seven) that the cells of the blood-islands, once they have clumped together, are irreversibly restricted to forming the progeny cell-types of the blood-islands only (i.e., endothelial cells and blood cells) (Pathway A of Fig. 8.1). This comes from the observation (Chapter Seven) that radioactively labelled blood-island fragments, transplanted into the AOV of unlabelled host blastoderms, tend to give rise only to endothelial cells and blood cells (Pathway A), with a negligible number contributing towards the formation of the coelomic linings (Pathway B of Fig. 8.1). The question as to when the final determination of the primary mesenchymal cells into blood-island cells and coelomic lining cells takes place, has still to be answered. The finding of Wilt (1974) that only the posterior regions of the unincubated chick blastoderm, cultured *in vitro*, are able to subsequently differentiate erythropoietically, lead Wilt (1974) to suggest that the determination of the presumptive erythropoietic tissue has already occurred by the time the egg is laid. As indicated in Chapter Seven, it would be of interest to determine whether at this early stage there are already two sub-populations of presumptive mesodermal cells, i.e., a sub-population of presumptive blood-island cells and sub-population of presumptive coelomic lining cells, or whether the two sub-populations only become discrete from each other at a later stage (e.g., as a result of environmental influences, such as contact with the ectoderm or endoderm).

2. The Proximo-Distal Polarity

The proximo-distal polarity in the morphogenesis of the yolk sac vascular system manifests itself in a centrifugal manner, both temporally and spatially, there being a graded sequence in the timing and nature of the developmental events in the AP and AOV. As the primary mesenchymal cells spread out in a fan-like manner from the central axis of the blastoderm (the primitive streak) towards the periphery, the morphogenesis of the yolk sac vascular system commences centripetally, occurring first in the posterior and posterio-lateral regions of the AOV, then in the anterior and medial parts of the AOV, and finally in the AP. Indeed, when the mesodermal cells in the posterior regions of the blastoderm are already embarking upon their overt differentiation pathways (commencing from stage 5-6), those in the anterior

and medial parts of the blastoderm are still in the process of migrating outward (see Chapter Two).

The question has been raised in the present study (Chapter Four) as to why the migratory mesodermal cells which move through the AP into the AOV do not clump together in the AP and differentiate erythropoietically there, but continue to migrate centrifugally into the AOV, where they then undergo their overt differentiation. In studying this question (Experiments 4.XII to 4.XV, Chapter Four), it has been found that the environment of the AP (i.e., the pellucida ectoderm and endoderm) is not conducive to mesodermal erythropoiesis. This comes from the observation that the stage 4 pellucida ectoderm, like the stage 4 opaca ectoderm, exerts an inhibitory effect on mesodermal erythropoiesis (Experiment 4.XIII, Chapter Four), and that the presence of the stage 4 pellucida endoderm, in contrast to that of the stage 4 opaca endoderm, is unable to counteract this inhibitory effect of the ectoderm on mesodermal erythropoiesis (Experiment 4.XIV, Chapter Four). Again this inhibitory effect is seen to be coupled with the tendency of the explants to spread out and become flattened in culture[1]. The significant finding from this study is that when the stage 4 pellucida mesoderm is isolated from the AP and cultured in combination with the stage 4 opaca endoderm (Experiment 4.XV, Chapter Four), its erythropoietic differentiation is dramatically enhanced to a level comparable with that of the stage 4 opaca mesoderm in combination with the stage 4 opaca endoderm (Experiment 4.XI, Chapter Four). It would thus appear that the stage 4 pellucida mesodermal cells are not stimulated to realize their overt differentiation until they subsequently migrate into the AOV and come into contact with the opaca endoderm.

The question then arises as to what causes these migratory mesenchymal cells, once they have entered the AOV, to stop migrating and to start to become organized into the components of the yolk sac vascular system, e.g., what causes the prospective blood-island cells to stop migrating and to start clumping together against the opaca endoderm? It would seem, in view of the finding (Chapter Four) that the very early

---

1. The question of whether the suppression of differentiation is a consequence of this spreading and/or flattening of the explants is discussed in Chapter Six, and on page 307 above).

(stage 1-2) opaca endoderm is not yet 'permissive' for mesodermal erytnropoiesis (Experiments 4.XVI to 4.XIX, Chapter Four), and in view furthermore of the report of Bennett (1973) that the yolk sac mesoderm itself exerts a stimulatory effect on the differentiation of the opaca endoderm, that the interactions between the opaca endoderm and the incoming mesoderm may be of vital importance for the endoderm, in turn, to acquire its permissive properties for stimulating the overt differentiation of the mesoderm. It would appear that the opaca endoderm is probably initially a surface conducive to the spreading (hence, migration) of the mesodermal cells, and only later, having interacted with the incoming mesoderm, acquires the properties conducive to the clumping together of the competent blood-island cells. This is in line with the histological observations (Chapter Two) that the mesodermal cells initially migrate into the AOV before they start to clump together against the opaca endoderm. Further supporting evidence in favour of this premise comes from the finding that whereas the presence of the stage 4 opaca endoderm (i.e., endoderm which has already come into contact with the migratory mesoderm) is able to overcome the tendency of the opaca ectoderm-plus-mesodermal explants to spread out and become flattened in culture (Experiment4.VIII, Chapter Four), the presence of the very early (stage 1-2) opaca endoderm (i.e., endoderm which has not yet come into contact with the migratory mesoderm) is unable to counteract the spreading of the explants in culture (Experiment 4.XVIII, Chapter Four).

The question has also been posed as to whether the synthesis and turnover of extracellular matrix components, such as collagen and hyaluronate, may play a causal role in the timing of the migration and subsequent clumping together of the primary mesenchymal cells (see Chapter Five). The finding (Experiments 5.VI to 5.VIII, Chapter Five) that the removal of the extracellular matrix component collagen, by means of a highly purified collagenase preparation (Sigma C-3267), does not reduce the erythropoietic differentiation of the isolated stage 4 opaca mesoderm cultured alone (Experiment 5.VI, Chapter Five), but appears to counteract the stimulatory influence of the opaca endoderm on mesodermal erythropoiesis (Experiments 5.VI and 5.VII), points to the importance of the integrity of the extracellular matrix for the occurrence of the necessary interactions between the mesoderm and the opaca endoderm for stimulating blood-island differentiation. That the

use of a less highly purified collagenase preparation, not specified to be free of non-specific protease and clostripain activities (Sigma C-6885), blocks mesodermal erythropoiesis but does not lyse or destroy the cells themselves (Experiments 5.VI to 5.VIII, Chapter Five), would suggest that the prime targets of the protease contaminants are the protein components at the cell-surface and in the extracellular matrix, again pointing to the importance of the extracellular components for cell differentiation.

According to the model of Toole and coworkers (see Toole *et al*, 1977), the synthesis of the extracellular matrix component hyaluronate is seen to be associated with cell migration, and its subsequent removal (due to hyaluronidase activity) is believed to be associated with cell differentiation. In this regard, Solursh (1976) and Fisher and Solursh (1977) have shown that hyaluronate is synthesized in the blastoderm during the period of mesodermal cell migration (stage 4), and that its experimental removal (by means of hyaluronidase treatment) leads to the clumping together of the mesodermal cells. This observation is significant in terms of the fact that the first overt sign of yolk sac vasculogenesis is the clumping together of the prospective blood-island cells (see Chapter Two). However, in the present preliminary study (Experiments 5.I to 5.V, Chapter Five) it is found that the removal of hyaluronate (by means of hyaluronidase treatment) has no effect on the erythropoietic differentiation of the mesodermal cells. On the other hand, one could argue that if hyaluronidase were indeed being produced by the blastoderm itself during the phase of overt differentiation (as suggested by the hypothesis of Toole *et al*, 1977), then the treatment of the explants with exogenous hyaluronidase (Experiments 5.I to 5.V, Chapter Five) would, at most, have speeded up the removal of hyaluronate. It would be relevant to compare the levels of hyaluronate and/or hyaluronidase in the AOV and AP during a stage in which the blood-island cells are already clumping together against the opaca endoderm, but are still migratory in the AP (i.e., stage 5-6).

In addition to this centripetally disposed gradient in the timing of the morphogenesis of the yolk sac vascular system, there is also a centripetally disposed gradation in the developmental properties exhibited by the components of this system. This is clearly demon-

strated, for example, in the relative amounts of blood cells formed in the different regions of the blastoderm (see Chapter Two). Thus, whereas in the posterior and posterio-lateral parts of the AOV the original blood-islands are relatively large, and differentiate into large sinusoidal blood vessels containing large numbers of intravascular blood cells, in the AP the original blood-islands (angioblasts) are relatively small and tend to give rise to empty blood vessels only. These conditions are found to grade into each other, there being an intermediate zone in the border region between the AOV and AP in which the blood-islands (angioblasts) tend to be larger than those in the rest of the AP, and to give rise to small numbers of intravascular blood cells as well.

This graded type of morphogenesis was earlier noted by Lillie (1919) in his textbook, and has now been re-emphasized and confirmed over 60 years later in the present study. During the intervening period, Lillie's overall perspective of the development of this system appears to have gone unnoticed in the numerous descriptive studies on this system (see below). Historically, it would appear that with the publication in 1917 and 1920 of Sabin's mammoth work on blood-island differentiation, in which she based her observations in the living AP but extrapolated them to encompass vasculogenesis in the AOV (see Section 1.2.3, Chapter One), workers have focused attention mainly on trying to discern the manner in which the blood-islands give rise to blood vessels and blood cells, and have either supported Sabin's observations (Sugiyama, 1926; Houser *et al*, 1961) or attempted to clear up the disparities introduced in consequence of Sabin's observations (e.g., Edmonds, 1964, 1966; Gonzalez-Crussi, 1971). With the advent of TEM, the current ideas of blood-island differentiation (see Section 1.2.2, Chapter One), were firmly re-established in studies such as Edmonds (1966) and Gonzalez-Crussi (1971), and many of Sabin's observations (e.g., the syncytial nature of the blood-islands; the total liquefaction of the central cells of the blood-islands; the endothelial origin of the blood cells) (see Section 1.2.3, Chapter One), were refuted. However, in view of this historical approach to the study of the subject, no paper since Lillie (1919) has taken a broader perspective in observing and comparing the cellular events which occur throughout the blastoderm (i.e., in the AOV and AP). Most studies have been concerned mainly with the cellular events in the AOV

only (e.g., Edmonds, 1964, 1966; Mato *et al*, 1964; Gonzalez-[illegible], 1971; Murphy and Carlson, 1978), with a few studies (e.g., [illegible] 1961, like Sabin, 1917, 1920) concentrating on the [illegible], and [illegible] observations to apply to events in the AOV.

[illegible] type of polarity in the extent of the formation of [illegible] has also been noted in the amphibian embryo, in which the erythropoietic activity is found to be limited to the more ventral [illegible] developing embryo (see Yamada, 1940; Fernand, 1943). [illegible] 1940) and Fernand (1943) believe this ventral location of the erythropoietic tissue to be a reflection of a dorso-ventral gradient in the amphibian embryo. According to this view, the erythropoietic tissue 'escapes' the dominant dorsalizing influence of the gradient by its location at a point far removed from the dorsal high point, and is able to differentiate unhindered by such external influences.

In the chick blastoderm the erythropoietic tissue can be considered to be similarly located, being situated ventrally and distally, in juxtaposition to the opaca endoderm, and far removed from the embryonic axis in the dorsal midline of the AP. Furthermore, observations that:

- the endoderm of the AOV is stimulatory for mesodermal erythropoiesis (Experiments in Sections 4.3.1, 4.3.2 and 4.3.3, Chapter Four; and the work of Wilt (1965a) and Miura and Wilt (1969));
- the ectoderm of both the AOV and AP exert an inhibitory effect on mesodermal erythropoiesis (Experiments in Sections 4.3.2 and 4.3.3. Chapter Four);
- the environment of the AP as a whole is unfavourable for mesodermal erythropoiesis (Experiments in Sections 3.3.1 and 3.3.2, Chapter Three; Experiments in Sections 4.3.1 and 4.3.3, Chapter Four). and
- some of the normally non-erythropoietic stage 8 pellucida mesodermal cells, transplanted into the stage 4 AOV, may differentiate erythropoietically (Experiment 3.I, Chapter Three);

provide circumstantial support for such a gradient, its effect being exerted both proximo-distally and dorso-ventrally (i.e., the inhibitory high point of the gradient emanating from the embryonic [illegible] proximo-dorsal region of the blastoderm, and the stimula-

tory high point emanating from the opaca endoderm [illegible]
and more distal region of the blastoderm). The findings, [illegible]
that the stage 4 opaca mesoderm, transplanted into the AP of [illegible]
host blastoderms, gives rise to large numbers of intravascular [illegible]
cells in the AP of the hosts (Experiment 3.II, Chapter Three) [illegible]
therefore unexpected, and would appear to cast doubt upon the validity [illegible]
of such an hypothesis. However, Finnegan (1953), in a similar type [illegible]
of experiment in the amphibian embryo, in which explants of the presumptive [illegible]
tial erythropoietic tissue were transplanted into the somatic region [illegible]
of the hosts, found that the transplanted tissue failed to differen-
tiate erythropoietically unless its underlying endoderm was present [illegible]
in the transplant. Finnegan (1953) suggested that ". . . the underly-
ing endoderm has supplied the erythropoietic tissue with the environ-
ment conducive to the development of red blood cells and has been
overcoming the adverse influence of the dorsal location." (Finnegan,
1953 p 391). It may well be that in the present study, in which
the opaca mesoderm of the chick blastoderm differentiated erythro-
poietically in the AP of the hosts (Experiment 3.II, Chapter Three),
that the transplanted mesoderm may have already spent sufficient time
in contact with the opaca endoderm to have become committed to differ-
entiate erythropoietically, regardless of its subsequent location in
the blastoderm. At present, further work examining this question of
the inhibitory properties of the AP are in progress.

Another contributing factor to the polarity in the type of hemato-
genesis observed in the AOV and AP may be tied in with the size dis-
tribution of the original blood-island clumps in these two regions.
As noted previously (see Chapter Two, and Lillie, 1919), the blood-
islands which form in the AOV are much larger than those which form in
the AP, and furnish material for the formation of both blood vessels
and blood cells. On the other hand, the blood-islands (angioblast)
in the AP are relatively small, and furnish material sufficient only
for the formation of empty blood vessels. These conditions grade into
each other. In terms of the inside-outside hypothesis of Weiss [illegible]
(discussed in more detail in Section 6.4, Chapter Six), the develop-
mental fates of the blood-island cells into endothelial cells or blood
cells would depend upon their positions in the blood-island. Accord-
ing to this hypothesis, gradients in metabolic activities [illegible]
with high points and low points at either the periphery or [illegible]

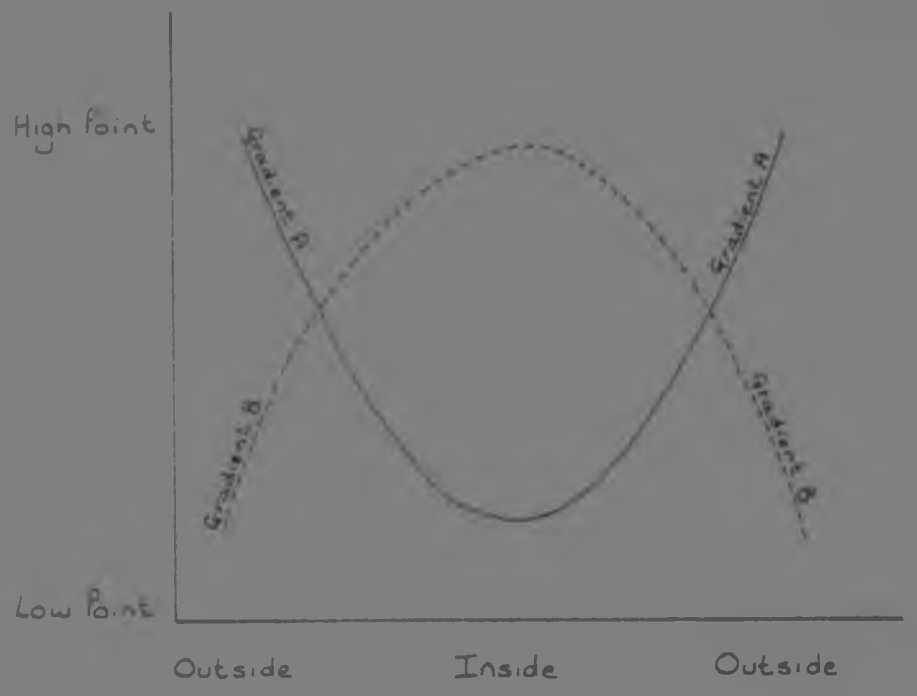


Figure 8.3

Diagram illustrating the two proposed gradients, with high points and low points at either the centre or the periphery of the blood-island clump, as hypothesized in the inside-outside model of Weiss (1953).

of the clump, are established in the blood-island. The one gradient (Gradient A in Fig. 8.3), set up in consequence of the course of reactions activated in the peripheral cells of the blood-island as a result of their interactions with the cells or extracellular materials surrounding the blood-island, will reflect the release of substances or activities spreading inward with a gradient from their source. The second gradient (Gradient B in Fig. 8.3), reflecting the concentration of diffusible metabolites produced by all the blood-island cells in the clump, will have a high point in the centre of the clump and will taper outwards towards the periphery. Depending upon their positions in the clump, therefore, the cells of the blood-island will be subjected to particular constellations of such factors, to which they will respond by particular differentiation steps. Since the concentrations of metabolites and activities at the centre or periphery of the clump will be at opposite extremes of the gradients

(Fig. 8.3), above or below certain threshold values, the cells in the centre or periphery of the blood-island will respond to these factors by differentiating into either blood cells or endothelial cells, respectively. As suggested by Weiss, ". . . if you cut the island in two, then a cell which used to be an inner one in the centre all of a sudden becomes an outer one, and that changes its future fate". (quoted from Waddington, 1959 page 11). Extending this further, one would expect that if one cut the blood-island into even smaller fragments so that all the cells became subjected to the concentration of metabolites and activities equal to or above the threshold values characteristic of the outside position (i.e., below the threshold values of the inside position), then all the cells would be expected to differentiate into vessel endothelium. This would correspond to the situation in the AP, in which the relatively small blood-islands (angioblasts) differentiate into empty blood vessels (see also Section 6.4, Chapter Six).

In this regard, a relevant parallel may be drawn between the development of the yolk sac blood-islands in the chick blastoderm, and the development of the early mouse embryo as a whole. Thus, whereas the outer and inner cells of the opaca blood-islands give rise to endothelial- and intravascular cells respectively, the outer and inner blastomeres of the early mouse embryo differentiate into the trophectoderm and the inner cell mass (ICM) respectively (see Gardner and Rossant, 1976). Furthermore, a number of studies on the determination of the developmental fates of these early mouse blastomeres have pointed to the importance of microenvironmental differences between the inner and outer cells in determining whether they will form the ICM or the trophectoderm (Tarkowski and Wróblewska, 1967; Hillman *et al*, 1972; see also review articles by Herbert and Graham, 1974; Gardner and Rossant, 1976).

Hillman *et al* (1972) constructed various arrangements of the blastomeres of cleavage-stage mouse embryos, in which ^{3}H-thymidine labelled- or genetically labelled cells were made to occupy either an inside or an outside position. These authors found a marked tendency for the 'outside' cells to colonize the trophoblast and later the trophectodermal tissue, and for the 'inside' cells to colonize the ICM and subsequently the foetal tissue. These observations strongly

support the inside-outside hypothesis as previously formulated for the mouse embryo by Tarkowski and Wróblewska (1967). According to this hypothesis, the blastomeres which remain in an external position throughout cleavage are thereby determined to form the trophectoderm, whereas those blastomeres which become wholly enclosed are thereby determined to form the ICM. This is analogous to the inside-outside hypothesis formulated by Weiss (1953) for the determination of the developmental fates of the chick blood-island cells.

The experiments carried out by Tarkowski and Wróblewska (1967) on the determination of the developmental fates of the early mouse blastomeres indicate also that the size of the morula is of importance in determining whether its cells will give rise to a normal blastocyst (i.e., the trophectoderm enclosing the ICM) or to a hollow trophoblastic vesicle only. These workers observed the *in vitro* development of isolated blastomeres taken from the 2- to the 8-cell stage embryo and found that the later the stage of the embryo from which the blastomeres were taken, the smaller the size of the morula produced, and the fewer the number of blastocysts formed. Thus, whereas the majority of blastomeres isolated from the 2-cell stage embryo developed into smaller, but normal blastocysts, only 30-40% of the blastomeres isolated from 4-cell stage embryos, and only 15% of the blastomeres isolated from 8-cell stage embryos, gave rise to true blastocysts. This decrease in the incidence of blastocysts was accompanied by an increase in the incidence of empty trophoblastic vesicles. These workers therefore proposed that the smaller the number of cells in the morula, the smaller the number of cells which could become enveloped by the other cells, thus the greater the potential of the cells to form trophoblastic cells.

Further evidence in favour of this proposal comes from the work of Stern (1973). This worker applied mechanical pressure to the early mouse embryo, forcing all the blastomeres to occupy an 'outside' position during cleavage. Under these conditions the cells were found to develop into trophoblast-like cells, the formation of the ICM cells being suppressed. This study of Stern (1973) is in line with the present study (Experiments 6.I to 6.III, Chapter Six), in which it has been shown that the flattening of the blastoderm explants by the application of physical pressure results in the suppression of the erythro-

poietic differentiation of the blood-island cells.

In terms of the present observations, and those of Lillie (1919), of the correlation between the size of the blood-islands in the AOV and AP and the types of development of the blood-island cells in these two regions, and in line with the inside-outside hypotheses of Weiss (1953) and Tarkowski and Wróblewski (1967), it may well be said that the size of the blood-islands (angioblasts) may be an important aspect in determining whether the cells will give rise to vessels containing blood cells, or to empty blood vessels only. Thus, the smaller the number of cells in the original blood-islands, the fewer the cells which will become enveloped by other cells, thus the greater the tendency of the cells to form vessel endothelium. This would result in a smaller number of blood cells being formed (as in the 'intermediate zone'). Finally, when the blood-islands became very small (as in the AP), then only endothelial cells would be formed.

An experiment which could be performed to test this proposal would be to observe the type of development which occurs when blood-island fragments of smaller and smaller sizes are implanted into explants composed of ectoderm and endoderm. This could be done either by implanting radioactively labelled blood-island fragments into unlabelled host explants, or by implanting quail blood-island fragments into chick host explants (or vice versa). After a period in culture, the type of differentiation of the different sizes of blood-islands could be ascertained from histological serial sections. The ectoderm-plus-endodermal explants could be obtained from both the AOV and AP, providing information not only about the influence of size on the erythropoietic differentiation of the blood-islands, but also on how the AP and AOV environments may affect such differentiation.

Finally, the question of why the blood-islands in the AOV should be larger than those in the AP has not been explained, but may well be tied in with the environmental influences inherent in these two regions. For example, whereas the opaca endoderm appears to be conducive to the clumping together of the blood-island cells, the pellucida endoderm may not possess such abilities, being an environment conducive rather to the spreading of the cells. Preliminary support for this contention comes from the observations that:

1. the prospective blood-island cells which migrate through the AP into the AOV do not form blood-island clumps in the AP, but rather continue to migrate centrifugally into the AOV where they subsequently clump together against the opaca endoderm (histological observations of Chapter Two);
2. whereas the presence of the stage 4 opaca endoderm is able to counteract the spreading tendencies of the stage 4 opaca ectoderm-plus-mesodermal explants in culture (Experiment 4.VIII, Chapter Four), the presence of the stage 4 pellucida endoderm does not prevent the *in vitro* spreading of the stage 4 pellucida ectoderm-plus-mesodermal explants (Experiment 4.XIV, Chapter Four).

It is probable that other factors, some of which have been discussed above (e.g., the synthesis and turnover of extracellular matrix materials; gradients in inhibitory and stimulatory influences; the ages and developmental histories of the mesodermal cells; and so on) may also be causally involved in the size distribution of the blood-island (or angioblastic) clumps.

8.2 Conclusion

The above discussion, in highlighting some questions and hypotheses concerning the dynamics of the creation of a simple three-dimensional pattern of tissue architecture, has illustrated how the integration of cellular interactions, both temporal and spatial, may mould the cellular behaviours appropriate for the morphogenesis of specific patterns of form. It is unlikely that single-factorial signals elicit these embryological responses. Rather, it would appear that numerous cytoplasmic and extracellular factors, dynamically co-ordinated in time and space, summate to produce the developmental programmes so clearly predictable from the fate maps of classical embryology. Whereas the ultimate responses of the cells to these multi-faceted cues must emanate primarily from the genome of the organism, and whereas these cues themselves must likewise originate primarily from the genome (e.g., at an earlier stage of development; some even during the stage of oocyte development), Biology is still relatively naive as to the precise series of molecular events involved in the transduction of this developmental information (see Britten and

Davidson, 1969; Davidson and Britten, 1973, 1979; Bautz and Karlson, 1973; Ptashne *et al*, 1976; Lash and Burger, 1977; Caplan and Ordahl, 1978; Ham and Veomett, 1980; Balinsky, 1981; and others). As so succinctly expressed by T.S. Eliot,

"We shall not cease from exploration
And the end of all our exploring
Will be to arrive where we started
And know the place for the first time."

(T.S. Eliot, 'Little Gidding').

REFERENCES

Abercrombie, M., 1980. The crawling movement of metazoan cells. Proc. R. Soc. Lond. B. Biol. Sci. 207, 129-147.

_____, and Heaysman, J.E.M., 1954. Observations on the social behaviour of cells in tissue culture. II. "Monolayering" of fibroblasts. Exp. Cell Res. 6, 293-306.

Adamson, E.D., 1981. The effect of collagen on cell division, cellular differentiation and embryonic development. (Manuscript for publication).

Alcantara, F. and Monk, M., 1974. Signal propagation during aggregation in the slime mould *Dictyostelium discoideum*. J. Gen. Microbiol. 85, 321-334.

Ali, I.U. and Hynes, R.O., 1978. Effects of LETS glycoprotein on cell motility. Cell 14, 439-446.

Balinsky, B.I., 1981. An Introduction to Embryology. Fifth edition. Saunders College Publishing Company, U.S.A.

_____, and Walther, H., 1961. The immigration of presumptive mesoblast from the primitive streak in the chick as studied with the electron microscope. Acta Embryol. Morphol. Exp. 4, 261-283.

Bank, A., Rifkind, R.A. and Marks, P.A., 1970. Regulation of globin synthesis. In: Gordon, A.S. (ed.), Regulation of Hematopoiesis. Vol. I, pages 701-729. Appleton-Century-Crofts, New York.

Barker, J.E., 1968. Development of the mouse hematopoietic system. I. Types of hemoglobin produced in embryonic yolk sac and liver. Dev. Biol. 18, 14-29.

Bautz, E.K.F. and Karlson, P., 1973. Regulation of Transcription and Translation in Eukaryotes. Springer-Verlag, New York.

Beaupain, D., Martin, C. and Dieterlen-Lièvre, F., 1979. Are developmental hemoglobin changes related to the origin of stem cells and site of erythropoiesis? Blood 53, 212-225.

Bellairs, R., 1971. Developmental Processes in Higher Vertebrates. Logos Press, Great Britain.

Bennett, N., 1973. Study of yolk-sac endoderm organogenesis in the chick using a specific enzyme (cysteine lyase) as a marker of cell differentiation. J. Embryol. Exp. Morphol. 29, 159-174.

Bernfield, M.R. and Banerjee, S.D., 1972. Acid mucopolysaccharide (glycosaminoglycan) at the epithelial-mesenchymal interface of mouse embryo salivary glands. J. Cell Biol. 52, 664-673.

_____, 1976. The basal lamina in epithelial-mesenchymal morphogenetic interactions. J. Gen. Physiol. 68, 1a.

Bernfield, M.R., Banerjee, S.D. and Conn, R.H., 1972. Dependence of salivary epithelial morphology and branching morphogenesis upon acid mucopolysaccharide-protein (proteoglycan) at the epithelial surface. J. Cell Biol. 52, 674-689.

_____, Cohn, R.H. and Banerjee, S.D., 1973. Glycosaminoglycans and epithelial organ formation. Am. Zool. 13, 1067-1083.

Bonner, J.T., 1947. Evidence for the formation of cell aggregates by chemotaxis in the development of the slime mould *Dictyostelium discoideum*. J. Exp. Zool. 106, 1-26.

_____, 1971. Aggregation and differentiation in the cellular slime molds. Annu. Rev. Microbiol. 25, 75-92.

Britten, R.J. and Davidson, E.H., 1969. Gene regulation for higher cells: a theory. Science 165, 349-357.

Brown, J.L. and Ingram, V.M., 1974. Structural studies on chick embryonic haemoglobins. J. Biol. Chem. 249, 3960-3972.

Bruns, G.A.P. and Ingram, V.M., 1973a. The erythroid cells and haemoglobins of the chick embryo. Trans. R. Soc. Lond. B. Biol. Sci. 266, 225-305.

_____, 1973b. Erythropoiesis in the developing chick embryo. Dev. Biol. 30, 455-459.

Cahn, R.D., 1968. Factors affecting inheritance and expression of differentiation: Some methods of analysis. In: Ursprung, H. (ed.), The Stability of the Differentiated State, pages 58-84. Springer-Verlag, Berlin, Heidelberg.

Campbell, G. Le M., Weintraub, H., Mayall, B.H. and Holtzer, H. 1971. Primitive erythropoiesis in early chick embryogenesis. II. Correlation between haemoglobin synthesis and the mitotic history. J. Cell Biol. 50, 669-681.

Caplan, A.I. and Ordahl, C.P., 1978. Irreversible gene repression model for control of development. Science 201, 120-130.

Carter, S.B., 1965. Principles of cell motility: the direction of cell movement and cancer invasion. Nature (Lond.) 208, 1183-1187.

_____, 1967. Effects of cytochalasins on mammalian cells. Nature (Lond.) 213, 261-264.

Chan, L.-N.L., 1976. Transport of globin mRNA from nucleus into cytoplasm in differentiating embryonic red blood cells. Nature (Lond.) 261, 157-159.

_____, and Ingram, V.M., 1973. Culture of erythropoietic cells from chick blastoderms. J. Cell Biol. 56, 861-365.

_____, Wiedmann, M. and Ingram, V.M., 1974. Regulation of specific gene expression during embryonic development: synthesis of globin messenger RNA during red cell formation in chick embryos. Dev. Biol. 40, 174-185.

Chen, L.B., Murray, A., Segal, R.A. and Walsh, M.L., 1978. Biological effects of intercellular LETS glycoprotein matrices. J. Cell Biol. 79, 157a.

Clawson, R.C. and Domm, L.V., 1969. Origin and early migration of primordial germ cells in the chick embryo: A study of the stages definitive primitive streak through eight somites. Am. J. Anat. 125, 87-112.

Crick, F., 1979. Split genes and RNA splicing. Science 204, 264-271.

Danchakoff, V., 1907. Über das erste Aufreten der Blutelemente im Hühnerembryo. Folia Haematol. Suppl. 4, 159-166. (Quoted by Sabin, 1920; Edmonds, 1966; Small and Davies, 1972).

_____ , 1908. Ueber die Blutbildung im Dottersack des Hühnchens. Anat. Gesellsch. Verhandl. 22, 72-81. (Quoted by Edmonds, 1966).

_____ , 1916a. Origin of the blood cells. Development of the haematopoietic organs and regeneration of the blood cells from the stand point of the monophyletic school. Anat. Rec. 10, 397-414.

_____ , 1916b. Concerning the conception of potentialities in the embryonic cells. Anat. Rec. 10, 415-416.

Davidson, E.H. and Britten, R.J., 1973. Organization, transcription, and regulation in the animal genome. Q. Rev. Biol. 48, 565-613.

_____ , 1979. Regulation of gene expression: possible role of repetitive sequences. Science 204, 1052-1059.

Derby, M.A., 1978. Analysis of glycosaminoglycans within the extracellular environments encountered by migrating neural crest cells. Dev. Biol. 66, 321-336.

_____ , Pintar, J.E. and Weston, J.A., 1978. Glycosaminoglycans and the development of the trunk neural crest. J. Gen. Physiol. 68, 4a.

Dessau, W., von der Mark, H., von der Mark, K. Fischer, S., 1980. Changes in the patterns of collagens and fibronectin during limb bud chondrogenesis. J. Embryol. Exp. Morphol. 57, 51-60

Dieterlen-Lievre, F., 1975. On the origin of the haemopoietic stem cells in the avian embryo: An experimental approach. J. Embryol. Exp. Morphol. 33, 607-619.

_____ , 1978. Yolk sac erythropoiesis. Experientia (Basel) 34, 284-289.

Ebendal, T., 1976. Migratory mesoblast cells in the young chick embryo examined by scanning electron microscopy. Zoon 4, 101-108.

Edmonds, R.H., 1964. Areas of attachment between developing blood cells. J. Ultrastruct. Res. 11, 577-580.

Edmonds, R.H., 1966. Electron microscopy of erythropoiesis in the avian yolk sac. Anat. Rec 154, 785-806.

Ekblom, P., Lash, J.W., Lehtonen, E., Nordling, S. and Saxen, L., 1979. Inhibition of morphogenetic cell interactions by 6-diazo-5-oxo-norleucine (DON). Exp. Cell Res. 121, 121-126.

_____ , Alitalo, K., Vaheri, A., Timpl, R. and Saxen, L., 1980. Induction of a basement membrane glycoprotein in embryonic kidney: possible role of laminin in morphogenesis. Proc. Nat. Acad. Sci. U.S.A. 77, 485-489.

Eyal-Giladi, H. and Kochav, S., 1976. From cleavage to primitive streak formation. A complementary normal table and a new look at the first stages of the development of the chick. I. General Morphology. Dev. Biol. 49, 321-337.

Fernald, R.L., 1943. The origin and development of the blood island of *Hyla regilla*. Univ. Calif. Publ. Zool. 51, 129-14. (Quoted by Finnegan, 1953).

Finch, R.A., Parker, C.L , and Walton, S.T., 1973. The lack of an inhibitory effect of hyaluronate on chondrogenesis in chick limb-bud mesoderm cells grown in culture. Cell Differ. 7, 283-293.

Finnegan, C.V., 1953. Studies of erythropoiesis in salamander embryos. J. Exp. Zool. 123, 371-395.

Fisher, M., and Solursh, M., 1977. Glycosaminoglycan localization and role in maintenance of tissue spaces in the early chick embryo. J. Embryol. Exp. Morphol. 42, 195-207.

_____ , 1979a. The influence of local environment on the organization of mesenchyme cells. J. Embryol. Exp. Morphol. 49, 295-306.

_____ , 1979b. The influence of the substratum on mesenchyme spreading in vitro. Exp. Cell Res. 123, 1-14.

Fitton-Jackson, S., 1967. Factors concerned with the maintenance of the synthetic balance of intercellular macromolecules. Biochem. J. 104, 13P.

Fleischmajer, R., and Billingham, R.E. (eds.), 1968. Epithelial-Mesenchymal Interactions. The Williams and Wilkins Company, Baltimore.

Folkman, J. and Moscona, A., 1978. Role of cell shape in growth control. Nature (Lond.) 273, 345-349.

Foster, M., Cook, R., Jr. and Stamas, T.A., 1956. In vitro studies of the effects of melanocyte population density on melanin formation. J. Embryol. Exp. Morphol. 122, 1-23.

Fujimoto, T., Ninomiya, T. and Ukeshima, A., 1976a. Observations of the primordial germ cells in blood samples from the chick embryo. Dev. Biol. 49, 278-282.

———— , Ukeshima, A. and Kiyofuji, R., 1976b. The origin, migration and morphology of the primordial germ cells in the chick embryo. Anat. Rec. 185, 139-154.

Gallera, J., 1965. Quelle est la durée necessaire pour déclencher des inductions neurales chez le poulet? Experientia (Basel) 21, 218-219.

———— , 1966. Le pouvoir inducteur de la chorde et du mésoblaste parachordal chez les oiseaux en fonction du facteur 'temps'. Acta Anat. 63, 388-397.

Gardner, R.L. and Rossant, J., 1976. Determination during embryogenesis. CIBA Found. Symp. 40, "Embryogenesis in Mammals"., pages 5-25. Elsevier North-Holland, Amsterdam.

Gerisch, G., 1968. Cell aggregation and differentiation in *Dictyostelium*. In: Moscona, A.A. and Monroy, A. (eds.), Curr. Top. Dev. Biol. 3, 157-197. Academic Press, New York.

Gingle, A.R., 1976. Critical density for relaying in *D. discoideum* and its relation to phosphodiesterase secretion into the extracellular medium. J. Cell Sci. 20, 1-20.

———— , 1977. cAMP enhanced aggregation of cells from early chick embryos. Dev. Biol. 58, 394-401.

———— , and Robertson, A., 1979. Responses of the early chick embryo to external cAMP sources. J. Embryol. Exp. Morphol. 53, 353-365.

Gonzalez-Crussi, F., 1971. Vasculogenesis in the chick embryo. An ultrastructural study. Am J. Anat. 130, 441-459.

Granholm, N.H. and Baker, J.R., 1970. Cytoplasmic microtubules and the mechanism of avian gastrulation. Dev. Biol. 23, 563-584.

Grobstein, C., 1959. Differentiation of vertebrate cells. In: Brachet, J. and Mirsky, A.E. (eds.), The Cell. Vol. I., pages 437-496. Academic Press, New York and London.

———— , 1965. Differentiation: Environmental factors, chemical and cellular. In: Willmer, E.N. (ed.), Cells and Tissues in Culture. Vol. I., pages 463-488. Academic Press, London and New York.

———— , and Cohen, J., 1965. Collagenase: Effect on the morphogenesis of embryonic salivary epithelium *in vitro*. Science 150, 626-628.

———— , and Zwilling, E., 1953. Modification of growth and differentiation of chorio-allantoic grafts of chick blastoderm pieces after cultivation at a glass-clot interface. J. Exp. Zool. 122, 259-284.

Gross, J.D., Peacey, M.J. and Trevan, D.J., 1976. Signal emission and signal propagation during early aggregation in [illegible] *discoideum*. J. Cell Sci. 22, 645-656.

Hagopian, H.K. and Ingram, V.M., 1971. Developmental changes of erythropoiesis in cultured chick blastoderms. J. Cell Biol. 51, 440-451.

———— , Lippke, J.A. and Ingram, V.M., 1972. Erythropoietic cell cultures from chick embryos. J. Cell Biol. 54, 98-106.

Ham, R.G. and Veomett, M.J., 1980. Mechanisms of Development. The C.V. Mosby Company, St. Louis, Toronto, London.

Hamburger, V. and Hamilton, H.L., 1951. A series of normal stages in the development of the chick embryo. J. Morphol. 88, 49-92.

Harris, A., 1973. Behaviour of cultured cells on substrata of variable adhesiveness. Exp. Cell Res. 77, 285-297.

Harrison, D.E. and Russell, E.S., 1972. Fetal liver erythropoiesis and yolk sac cells. Science 177, 187.

Harrison, R.G., 1914. The reaction of embryonic cells to solid structure. J. Exp. Zool. 17, 521-544.

Hay, E.D., 1968. Organization and fine structure of epithelium and mesenchyme in the developing chick embryo. In: Fleischmajer, R. and Billingham, R.E. (eds.), Epithelial-Mesenchymal Interactions., pages 31-55. The Williams and Wilkins Company, Baltimore.

———— , 1973. Origin and role of collagen in the embryo. Am. Zool. 13, 1085-1107.

———— , 1977. Interaction between the cell surface and extracellular matrix in corneal development. In: Lash, J.W. and Burger, M.M. (eds.), Cell and Tissue Interactions., pages 115-137. Soc. Gen. Physiol. Ser. 32, Raven Press, New York.

———— , and Meier, S., 1974. Glycosaminoglycan synthesis by embryonic inductors: neural tube, notochord and lens. J. Cell Biol. 62, 889-898.

Hell, A., 1964a. The initial synthesis of haemoglobin in de-embryonated chick blastoderms. I. Metabolism of the blastodisc cultured in vitro. J. Embryol. Exp. Morphol. 12, 609-619.

———— , 1964b. The initial synthesis of haemoglobin in de-embryonated chick blastoderms. II. The effect of metabolic inhibitors on the blastodisc cultured in vitro. J. Embryol. Exp. Morphol. 12, 621-632.

Herbert, M.C. and Graham, C.F., 1974. Cell determination and biochemical differentiation of the early mammalian embryo. In Moscona, A.A. and Monroy, A. (eds.), Curr. Top. Dev. Biol. 8, 151-178. Academic Press, New York and London.

Hilfer, S.R., 1962. The stability of embryonic chick thyroid cells *in vitro* as judged by morphological and physiological criteria. Dev. Biol. 4, 1-21.

Hillman, N., Sherman, M.I. and Graham, C., 1972. The effect of spatial arrangement on cell determination during mouse development. J. Embryol. Exp. Morpnol. 28, 263-278.

Holtfreter, J., 1943. A study of the mechanics of gastrulation. Part I. J. Exp. Zool. 94, 261-318.

_____ , and Hamburger, V., 1955. Embryogenesis: Progressive differentiation. Amphibians. In: Willier, B.H., Weiss, P.A. and Hamburger, V. (eds.), Analysis of Development., pages 230-296. W.B. Saunders Company, Philadelphia and London.

Holtzer, H., Abbott, J., Lash, J. and Holtzer, S., 1960. The loss of phenotypic traits by differentiated cells in vitro. I. Dedifferentiation of cartilage cells. Proc. Nat. Acad. Sci. U.S.A. 46, 1533-1542.

Houser, J.W., Ackerman, G.A. and Knouff, R.A., 1961. Vasculogenesis and erythropoiesis in the living yolk sac of the chick embryo. A phase microscopic study. Anat. Rec. 140, 29-43.

Houssaint, E., Belo, M. and Le Douarin, N.M., 1976. Investigations on cell lineage and tissue interactions in the developing bursa of Fabricius through interspecific chimaeras. Dev. Biol. 53, 250-264.

Hughes, A.F.W., 1943. The blood pressure of the chick embryo during development. J. Exp. Biol. 19. 232-237.

Humason, G.L., 1967. Animal Tissue Techniques. Second Edition. W.H. Freeman and Company, San Francisco and London.

Hunt, T.E., 1937. The origin of entodermal cells from the primitive streak of the chick embryo. Anat. Rec. 68, 449-460.

Hyer, B.J. and Chan L.-N.L., 1978. Initial synthesis of globin peptide chains in differentiating embryonic red blood cells. Dev. Biol. 66, 279-284.

Ingram, V.M., Chan, L.-N.L., Hagopian, H.K., Lippke, J.A. and Wu, L., 1974. Differentiation in the early chick embryo: effects of bromodeoxyuridine on erythropoiesis. Dev. Biol. 36, 411-427.

Jotereau, F. and Houssaint, E., 1977. Experimental studies on the migration and differentiation of primary lymphoid stem cells in the avian embryo. In: Solomon, J.E. and Horton, J.D. (eds.), Developmental Immunobiology. Elsevier North-Holland Biomedical Press, Amsterdam.
(Quoted by Martin *et al*, 1978, 1979).

Kallman, F. and Grobstein, C., 1965. Source of collagen at epithelio-mesenchymal interfaces during inductive interaction. Dev. Biol. 11, 169-183.

Kefalides, N.A., 1971. Isolation of a collagen from basement membranes containing three identical α-chains. Biochem. Biophys. Res. Commun. 45, 226-234.

Kinniburgh, A.J., Mertz, J.E. and Ross, J., 1978. The precursors of mouse β-globin messenger RNA contains two intervening RNA sequences. Cell 14, 681-693.

Kosher, R.A. and Church, R.L., 1975. Stimulation of *in vitro* somite chondrogenesis by procollagen and collagen. Nature (Lond.) 258, 327-330.

Kosher, R.A., Lash, J.W. and Minor, R.R., 1973. Environmental enhancement of *in vitro* chondrogenesis. IV. Stimulation of somite chondrogenesis by exogenous chondromucoprotein. Dev. Biol. 35, 210-220.

Kresina, T.F. and Miller, E.J., 1979. Isolation and characterization of basement membrane collagen from human placental tissue: Evidence for the presence of two genetically distinct collagen chains. Biochemistry 18, 3089-3097.

La Brecque, D.R. and Howard, R.B., 1976. The preparation and characterization of intact isolated parenchymal cells from rat liver. In: Prescott, D.M. (ed.), Methods in Cell Biology. Vol. XIV. pages 327-340. Academic Press, New York, San Francisco and London.

Lash, J.W. and Burger, M.M. (eds.), 1977. Cell and Tissue Interactions. Soc. Gen. Physiol. Ser. 32, Raven Press, New York.

———— , and Vasan, N.S., 1977. Tissue interactions and extracellular matrix components. In: Lash, J.W. and Burger, M.M. (eds.), Cell and Tissue Interactions, pages 101-113. Soc. Gen. Physiol. Ser. 32, Raven Press, New York.

———————————— , 1978. Somite chondrogenesis *in vitro*. Stimulation by exogenous extracellular matrix components. Dev. Biol. 66, 151-171.

Lassila, O., Eskola, J., Toivanen, P., Martin, C. and Dieterlen-Lievre, F., 1978. The origin of lymphoid stem cells studied in chick yolk sac-embryo chimaeras. Nature (Lond.) 272, 353-354.

———————————— , and Dieterlen-Lievre, F., 1980. Lympnoid stem cells in the intraembryonic mesenchyme of the chicken. Scand. J. Immunol. 11, 445-448.

Laurent, T.C., 1970. Structure of hyaluronic acid. In: Balazs, E.A. (ed.), Chemistry and Molecular Biology of the Intercellular Matrix. Vol. 2., pages 703-732 Academic Press, New York.

Le Douarin, N., 1973a. A biological cell labelling technique and its use in experimental embryology. Dev. Biol. 30, 217-222.

Le Douarin, N., 1973b. A Feulgen-positive nucleolus. Exp. Cell Res. 77, 459-468.

_____, 1980. The ontogeny of the neural crest in avian embryo chimaeras. Nature (Lond.) 286, 663-669.

_____, Houssaint, E., Jotereau, F. and Belo, M., 1975. Origin of haemopoietic stem cells in the embryonic bursa of Fabricius and bone marrow studied through interspecific chimaeras. Proc. Nat. Acad. Sci. U.S.A. 72, 2701-2705.

_____, and Jotereau, F., 1973. Origin and renewal of lymphocytes in avian embryo thymuses. Nature New Biol. 246, 25-27

_____, 1975. Tracing of cells of the avian thymus through embryonic life in interspecific chimeras. J. Exp. Med. 142, 17-40.

Lee, H.-Y., Nagele, R.G. and Goldstein, M.M., 1978. Scanning electron microscopy of primordial germ cells in early chick embryos. J. Exp. Zool. 206, 457-462.

Lillie, F.R., 1919. The Development of the Chick. Second Edition. Henry Holt and Company, New York.

Linsenmayer, T.F., Trelstad, R.L. and Gross, J., 1973. The collagen of chick embryonic notochord. Biochem. Biophys. Res. Commun. 53, 39-45.

Loeb, L., 1921. Amoeboid movement, tissue formation and consistency of protoplasm. Am. J. Physiol. 56, 140-167.

Loomis, W.F., 1979. Biochemistry of aggregation of *Dictyostelium*. A Review. Dev. Biol. 70, 1-12.

Lucas, A.M. and Jamroz, C., 1961. Atlas of Avian Hematology. Agric. Monogr. 25, United States Department of Agriculture, Washington.

Mahoney, K.A., Hyer, B.J. and Chan, L.-N.L., 1977. Separation of primitive and definitive erythroid cells of the chick embryo. Dev. Biol. 56, 412-416.

Manasek, F.J., 1975. The extracellular matrix: A dynamic component of the developing embryo. In: Moscona, A.A. and Monroy, A. (eds.), Curr. Top. Dev. Biol. 10, 35-102. Academic Press, New York.

_____, Reid, M., Vinson, W., Seyer, J. and Johnson, R., 1973. Glycosaminoglycan synthesis by the early embryonic chick heart. Dev. Biol. 35, 332-348.

Marks, P.A. and Rifkind, R.A., 1972a. Protein synthesis: Its control in erythropoiesis. Science 175, 955-961.

_____, 1972b. Fetal liver erythropoiesis and yolk sac cells. Science 177, 187.

Markwald, R.R., Fitzharris, T.P. and Adams-Smith, W.N., 1975. Structural analysis of endocardial cytodifferentiation. Dev. Biol. 42, 160-180.

_____ , and Manasek, F.J., 1977. Structural development of endocardial cushions. Am. J. Anat. 148, 85-120.

Martin, C., Beaupain, D. and Dieterlen-Lièvre, F., 1978. Developmental relationships between vitelline and intra-embryonic haemopoiesis studied in avian 'yolk sac chimaeras'. Cell Differ. 7, 115-130.

_____ , 1980. A study of the development of the haemopoietic system using quail-chick chimeras obtained by blastoderm recombination. Dev. Biol. 75, 303-314.

_____ , Lassila, O., Nurmi, T., Eskola, J., Dieterlen-Lièvre, F. and Toivanen, P., 1979. Intraembryonic origin of lymphoid stem cells in the chicken: Studies with sex chromosome and IgG allotype markers in histocompatible yolk sac-embryo chimaeras. Scand. J. Immunol. 10, 333-338.

Mato, M., Aikawa, E. and Kishi, K., 1964. Some observations on interstice between mesoderm and endoderm in the area vasculosa of chick blastoderm. Exp. Cell Res. 35, 426-428.

Medoff, J. and Zwilling, E., 1972. Appearance of myosin in the chick limb bud. Dev. Biol. 28, 138-141.

Meier, S. and Hay, E.D., 1974a. Control of corneal differentiation by extracellular materials. Collagen as a promoter and stabilizer of epithelial stroma production. Dev. Biol. 38, 249-270.

_____ , 1974b. Stimulation of extracellular matrix synthesis in the developing cornea by glycosaminoglycans. Proc. Nat. Acad. Sci. U.S.A. 71, 2310-2313.

Mercer, E.H. and Birbeck, M.S.C., 1972. Electron Microscopy. A Handbook for Biologists. Third Edition Blackwell Scientific Publications, Osney Mead, Oxford.

Metcalf, D. and Moore, M.A.S., 1971. Haemopoietic Cells. North-Holland Publishing Company, Amsterdam and London.

Meyer, D.B., 1964. The migration of primordial germ cells in the chick embryo. Dev. Biol. 10, 154-190.

Miller, E.J., 1971. Isolation and characterization of a collagen from chick cartilage containing three identical α-chains. Biochemistry 10, 1652-1659.

_____ , 1977. The collagens of the extracellular matrix. In: Lash, J.W. and Burger, M.M. (eds.), Cell and Tissue Interactions, pages 71-86. Soc. Gen. Physiol. Ser. 32, Raven Press, New York.

Miller, E.J. and Mathews, M.B., 1974. Characterization of notochord collagen as a cartilage type collagen. Biochem. Biophys. Res. Commun. 60, 424-430.

_____ , and Matukas, V.J., 1974. The biosynthesis of collagen. Fed. Proc. 33, 1197-1204.

Miura, Y., Sawatani, S., Terasawa, T. and Masudu, R., 1979. Enhancement of heme synthesis in quail embryo yolk sac cells by highly polar compounds. Blood 54, 421-428.

_____ , Terasawa, T. and Sawatani, S., 1976. Dimethyl sulfoxide (DMSO) stimulates heme synthesis in quail embryonic yolk sac cells. Exp. Cell Res. 99, 197-200.

_____ , and Wilt, F.H., 1969. Tissue interaction and the formation of the first erythroblasts of the chick embryo. Dev. Biol. 19, 201-211.

_____ , 1970. The formations of blood islands in dissociated-reaggregated chick embryo yolk sac cells. Exp. Cell Res. 59, 217-226.

Moore, M.A.S. and Metcalf, D., 1970. Ontogeny of the haemopoietic system: Yolk sac origin of *in vivo* and *in vitro* colony forming cells in the developing mouse embryo. Br. J. Haematol. 18, 279-296.

_____ , and Owen, J.J.T., 1965. Chromosome marker studies on the development of the haemopoietic system in the chick embryo. Nature (Lond.) 208, 956-990.

_____ _____ , 1967a. Chromosome marker studies in the irradiated chick embryo. Nature (Lond.) 215, 1081-1082.

_____ , 1967b. Stem-cell migration in developing myeloid and lymphoid systems. Lancet ii, 658-659.

Morriss, G.M. and Solursh, M., 1978. Regional differences in mesenchymal cell morphology and glycosaminoglycans in early neural-fold stage rat embryos. J. Embryol. Exp. Morphol. 46, 37-52.

Muchmore, W.B., 1951. Differentiation of the trunk mesoderm in *Amblystoma maculatum*. J. Exp. Zool. 118, 137-185.

Muir, H., 1977. Structure and function of proteoglycans of cartilage and cell-matrix interactions. In: Lash, J.W. and Burger, M.M. (eds.), Cell and Tissue Interactions., pages 87-99. Soc. Gen. Physiol. Ser. 32, Raven Press, New York.

Mulherkar, L., 1958. Induction by regions lateral to the streak in the chick embryo. J. Embryol. Exp. Morphol. 6, 1-14.

Murphy, M.E. and Carlson, E.C., 1978. An ultrastructural study of developing extracellular matrix in vitelline blood vessels of the early chick embryo. Am. J. Anat. 151, 345-376.

Murray, P.D.E., 1932. The development *in vitro* of the blood of the early chick embryo. Proc. R. Soc. Lond. B. Biol. Sci. III, 497-521.

Nevo, Z. and Dorfman, A., 1972. Stimulation of chondromucoprotein synthesis in chondrocytes by extracellular chondromucoprotein. Proc. Nat. Acad. Sci. U.S.A. 69, 2069-2072.

New, D.A.T., 1955. A new technique for the cultivation of the chick embryo *in vitro*. J. Embryol. Exp. Morphol. 3, 320-331.

Nicolet, G., 1967. Le chronologie d'invagination chez le Poulet: Etude à l'aide de la thymidine tritiée. Experientia (Basel) 23, 576-577.

_____, 1970. Analyse autoradiographique de la localisation des différentes ébauches présomptives dans la ligne primitive de l'embryon de Poulet. J. Embryol. Exp. Morphol. 23, 79-108.

O'Brien, R.A., 1961. Development of haemoglobin by de-embryonated chick blastoderms cultured *in vitro* and the effect of abnormal RNA upon its synthesis. J. Embryol. Exp. Morphol. 9, 202-221.

Orkin, R.W. and Toole, B.P., 1978. Hyaluronidase activity and hyaluronate content of the developing chick embryo heart. Dev. Biol. 66, 308-320.

Owen, J.J.T. and Ritter, M.A., 1969. Tissue interaction in the development of thymus lymphocytes. J. Exp. Med. 129, 431-437.

Parker, R.E., 1979. Introductory Statistics for Biology. Second Edition. Inst. Biol. Stud. Biol. [illegible], Edward Arnold Ltd., London.

Patten, B.M., 1971. Early Embryology of the Chick. Fifth Edition. McGraw-Hill Book Company, U.S.A.

_____, and Carlson, B.M., 1974. Foundations of Embryology. Third Edition. McGraw-Hill Book Company, U.S.A.

Pearlstein, E., 1976. Plasma membrane glycoprotein which mediates adhesion of fibroblasts to collagen. Nature (Lond.) 262, 497-500.

Pennypacker, J.P., Hassell, J.R., Yamada, K.M. and Pratt, R.M., 1979. The influence of an adhesive cell surface protein on chondrogenic expression *in vitro*. Exp. Cell Res. 121, 411-415.

Pintar, J.E., 1978. Distribution and synthesis of glycosaminoglycans during quail neural crest morphogenesis. Dev. Biol. 67, 444-464.

Pouyssegur, J., Willingham, M. and Pastan, I., 1977. Role of cell surface carbohydrates and proteins in cell behaviour: Studies on the biochemical reversion of an N-acetylglucosamine deficient fibroblast mutant. Proc. Nat. Acad. Sci. U.S.A. 74, 243-247.

Pratt, R.M., Larsen, M.A. and Johnston, M.C., 1975. Migration of cranial neural crest cells in a cell-free hyaluronate-rich matrix. Dev. Biol. 44, 298-305.

_____ . Morriss, G. and Johnston, M., 1978. The source, distribution, and possible role of hyaluronate in the migration of chick cranial neural crest cells. J. Gen. Physiol. 68, 15a-16a.

Ptashne, M., Backman, K., Humayun, M.Z., Jeffrey, A., Maurer, R., Meyer, B. and Sauer, R.T., 1976. Autoregulation and function of a repressor in basteriophage lambda. Science 194, 156-161.

Ramsey, W.S., 1972a. Analysis of individual leucocyte behaviour during chemotaxis. Exp. Cell Res. 70, 129-139.

_____ , 1972b. Locomotion of human polymorphonuclear leucocytes. Exp. Cell Res. 72, 489-501.

Reporter, M. and Rosenquist, G.C., 1972. Adenosine 3',5'-monophosphate: Regional differences in chick embryos at the head process stage. Science 178, 628-630.

Revel, J.P., 1974. Some aspects of cellular interactions in development. In: Moscona, A.A. (ed.), The Cell Surface in Development, pages 51-65. John Wiley and Sons, New York.

_____ , and Solursh, M., 1978. Ultrastructure of primary mesenchyme in chick and rat embryos. Scanning Electron Micros. II, 1040-1046.

Rhodes, R.K. and Miller, E.J., 1978. Physiochemical characterization and molecular organization of collagen A and B chains. Biochemistry 17, 3442-3448.

Rifkind, R.A., Chui, D. and Epler, H., 1969. An ultrastructural study of early morphogenetic events during the establishment of fetal hepatic erythropoiesis. J. Cell Biol. 40, 343-365.

Robertson, A., 1977. (Quoted in Boiteux, A., Hess, B., Plesser, Th. and Murray, J.D., FEBS (Fed. Eur. Biochem. Soc.) Lett. 77, 1-4.)

_____ , and Cohen, M.H., 1974. Quantitative analysis of the development of the cellular slime moulds. II. Lect. Math. Life Sci. 6, 44-62.

_____ , Drage, D.J. and Cohen, M.H., 1972. Control of aggregation in *Dictyostelium discoideum* by an external periodic pulse of cyclic adenosire monophosphate. Science 175, 333-335.

_____ , and Gingle, A.R., 1977. Axial bending in the early chick embryo by a cyclic adenosine monophosphate source. Science 197, 1078-1079.

_____ , Grutsch, J.F. and Gingle, A.R., 1978. Cyclic adenosine monophosphate production by embryonic chick cells. Science 199, 990-991.

Rodewald, R. and Karnovsky, M.J., 1974. Porous substructure of the glomerular slit diaphragm in the rat and mouse. J. Cell Biol. 60, 423-433.

Rogers, A.W., 1969. Techniques in Autoradiography. Elsevier North-Holland, Amsterdam.

Romanoff, A.L., 1960. The Avian Embryo. The Macmillan Company, New York.

Roscoe, J.T., 1975. Fundamental Research Statistics for the Behavioral Sciences. Second Edition. Holt, Rinehart and Winston, Inc., U.S.A.

Rosenquist, G.C., 1966. A radioautographic study of labeled grafts in the chick blastoderm. Development from primitive-streak stages to stage 12. Carnegie Inst. Wash. Publ. 38, 71-110.

Rugh, R., 1962. Experimental Embryology. Third Edition. Burgess Publishing Company, Minneapolis.

Sabin, F.R., 1917. Preliminary note on the differentiation of angioblasts and the method by which they produce blood-vessels, blood-plasma and red blood-cells as seen in the living chick. Anat. Rec. 13, 199-204.

_____ , 1920. Studies on the origin of blood-vessels and of red blood-corpuscles as seen in the living blastoderm of chicks during the second day of incubation. Carnegie Inst. Wash. Publ. 9, 213-262.

Samarut, J., 1979. Erythropoietin-like factor production by young chick blastoderms. Dev. Biol. 70, 278-282.

Saxén, L. and Toivonen, S., 1962. Primary Embryonic Induction. Logos Press, London.

Searls, R.L., 1965. An autoradiographic study of the uptake of S^{35}-sulfate during the differentiation of limb bud cartilage. Dev. Biol. 11, 155-168.

Settle, G.W., 1954. Localization of the erythrocyte-forming areas in the early chick blastoderm cultivated *in vitro*. Carnegie Inst. Wash. Publ. 35, 221-235

Small, J.V. and Davies, H.G., 1972. Erythropoiesis in the yolk sac of the early chick embryo: An electron microscope and micro-spectrophotometric study. Tissue and Cell 4, 341-378.

Solursh, M., 1976. Glycosaminoglycan synthesis in the chick gastrula. Dev. Biol. 50, 525-530.

_____ , and Revel, J.P., 1978. A scanning electron microscope study of cell shape and cell appendages in the primitive streak region of the rat and chick embryo. Differentiation 11, 185-190.

Solursh, M., Vaerewyck, S.A. and Reiter, R.S., 1974. Depression by hyaluronic acid of glycosaminoglycan synthesis by cultured chick embryo chondrocytes. Dev. Biol. 41, 233-244.

Spemann, H., 1919. Experimentelle Forschungen zum Determinations- und Individualitätsproblem. Naturwissenschaften 32, 1-33.

_____, 1921. Die Erzeugung tierischer Chimären durch heteroplatische embryonale Transplantation Zwischen *Triton cristatus* und *taeniatus*. Wilhelm Roux' Arch. Entwicklungsmech. Org. 48, 533-570.
(Quoted by Balinsky, 1981).

_____, 1938. Embryonic Development and Induction. Yale University Press, New Haven.

Spratt, N.T. and Haas, H., 1961. Integrative mechanisms in development of the early chick blastoderm. III. Role of cell population size and growth potentiality in synthetic systems larger than normal. J. Exp. Zool. 147, 271-293.

Stern, M.S., 1973. Development of cleaving mouse embryos under pressure. Differentiation 1, 407-412.

Sugiyama, S., 1926. Origin of thrombocytes and of the different types of blood-cells as seen in the living chick blastoderm. Carnegie Inst. Wash. Publ. 97, 121-147.

Swift, C.H., 1914. Origin and early history of the primordial germ cells in the chick. Am. J. Anat. 15, 483-516.

Tarkowski, A.K. and Wróblewska, J., 1967. Development of blastomeres of mouse eggs isolated at the 4- and 8-cell stage. J. Embryol. Exp. Morphol. 18, 155-180.

Thesleff, I., Barrach, H.J., Foidart, J.M., Vaheri, A., Pratt, R.M. and Martin, G.R., 1981. Changes in the distribution of type IV collagen, laminin, proteoglycan, and fibronectin during mouse tooth development. Dev. Biol. 81, 182-192.

_____, Stenman, S., Vaheri, A. and Timpl, R., 1979. Changes in the matrix proteins, fibronectin and collagen, during differentiation of mouse tooth germ. Dev. Biol. 70, 116-126.

Tilghman, S.M., Tiemeier, D.C., Seidman, J.G., Peterlin, B.M., Sullivan, M., Maizel, J.V. and Leder, P., 1978a. Intervening sequence of DNA identified in the structural portion of a mouse β-globin gene. Proc. Nat. Acad. Sci. U.S.A. 75, 725-729.

_____, Curtis, P.J., Tiemeier, D.C., Leder, P. and Weissman, C., 1978b The intervening sequence of a mouse β-globin gene is transcribed within the 15S β-globin mRNA precursor. Proc. Nat. Acad. Sci. U.S.A. 75, 1309-1313.

Toole, B.P., 1972. Hyaluronate turnover during chondrogenesis in the developing chick limb and axial skeleton. Dev. Biol. 29, 321-329.

———— , 1973a. Hyaluronate and hyaluronidase in morphogenesis and differentiation. Am. Zool. 13, 1061-1065.

———— , 1973b. Hyaluronate inhibition of chondrogenesis: Antagonism of thyroxine, growth hormone, and calcitonin. Science 180, 302-303.

———— , and Gross, J., 1971. The extracellular matrix of the regenerating newt limb: Synthesis and removal of hyaluronate prior to differentiation. Dev. Biol. 25, 57-77.

———— , Jackson, G. and Gross, J , 1972. Hyaluronate in morphogenesis: Inhibition of chondrogenesis *in vitro*. Proc. Nat. Acad. Sci. U.S.A. 69, 1384-1386.

———— , Okayama, M., Orkin, R.W., Yoshimura, M., Muto, M. and Kaji, A., 1977. Developmental roles of hyaluronate and chondroitin sulfate proteoglycans. In: Lash, J.W. and Burger, M.M. (eds.), Cell and Tissue Interactions, pages 139-154. Soc. Gen. Physiol. Ser. 32, Raven Press, New York.

———— , and Trelstad, R.L., 1971. Hyaluronate production and removal during corneal development in the chick. Dev. Biol. 26, 28-35.

Trelstad, R.L., Hay, E.D. and Revel, J.P., 1967. Cell contact during early morphogenesis in the chick. Dev. Biol. 16, 78-106.

Trentin, J.J., 1970. Influence of hematopoietic organ stroma (hematopoietic inductive microenvironments) on stem cell differentiation. In: Gordon, A.S. (ed.), Regulation of Hematopoiesis. Vol. I., pages 161-186. Appleton-Century-Crofts, New York.

Trinkaus, J.P., 1969. Cells into Organs. Prentice-Hall Inc., Englewood Cliffs, New Jersey.

———— , 1976. On the mechanism of metazoan cell movements. In: Poste, G. and Nicolson, G.L. (eds.), The Cell Surface in Animal Embryogenesis and Development, pages 225-329 Elsevier Biomedical Press, North-Holland.

Von der Mark, H., von der Mark, K. and Gay, S., 1976a. Study of differential collagen synthesis during development of the chick embryo by immunofluorescence. I. Preparation of collagen type I and type II specific antibodies and their application to early stages of the chick embryo. Dev. Biol. 48, 237-249.

von der Mark, K., von der Mark, H. and Gay, S., 1976b. Study of differential collagen synthesis during development of the chick embryo by immunofluorescence. II. Localization of type I and type II collagen during long bone development. Dev. Biol. 53, 153-170.

Waddington, C.H., 1959. The nature of biological organization. In: Waddington, C.H. (ed.), Biological Organization, Cellular and Subcellular., pages 1-21. Proceedings of a Symposium. Pergamon Press, London.

_____ , and Schmidt, G.A., 1933. Induction by heteroplastic grafts of the primitive streak in birds. Wilhelm Roux' Arch. Entwicklungsmech Org. 128 522-563.

Wainwright, S.D., and Wainwright, L.K., 1979. Stimulation by isoprenaline of erythropoiesis in cultures of dispersed cells of the primitive-streak chick blastodisc. Exp. Hematol. 7, 152-165.

Wakely, J. and England, M.A., 1977. Scanning electron microscopy (SEM) of the chick embryo primitive streak. Differentiation 7, 181-186.

_____ , 1979. Scanning electron microscopical and histochemical study of the structure and function of basement membranes in the early chick embryo. Proc. R. Soc. Lond. B. Biol. Sci. 206, 329-352.

Weintraub, H., Campbell, G. Le M. and Holtzer, H., 1971. Primitive erythropoiesis in early chick embryogeresis. I. Cell cycle kinetics and control of cell division. J. Cell Biol. 50, 652-668.

Weiss, P.A., 1945. Experiments on cell and axon orientation *in vitro*: the role of colloidal exudates in tissue organization. J. Exp. Zool. 100, 353-386.

_____ , 1953. Some introductory remarks on the cellular basis of differentiation. J. Embryol. Exp. Morphol. 1, 181-211.

_____ , 1961. Guiding principles in cell locomotion and aggregation. Exp. Cell Res. Suppl. 8, 260-281.

Wessells, N.K. and Cohen, J., 1968. Effects of collagenase on developing epithelia *in vitro*: lung, ureteric bud and pancreas. Dev. Biol. 18, 294-309.

Whittaker, J.R., 1963. Changes in melanogeresis during the dedifferentiation of chick retinal pigment cells in cell culture. Dev. Biol. 8, 99-127.

Wiebkin, O.W. and Muir, H., 1973. The inhibition of sulphate incorporation in isolated adult chondrocytes by hyaluronic acid. FEBS (Fed. Eur. Biochem. Soc.) Lett. 37, 42-46.

Wilt, F.H., 1962. The ontogeny of chick embryo hemoglobin. Proc. Nat. Acad. Sci. U.S.A. 48, 1582-1590.

_____ , 1965a Erythropoiesis in the chick embryo: The role of endoderm. Science 147, 1588-1590.

Wilt, F.H., 1965b. Regulation of the initiation of chick embryo hemoglobin synthesis. J. Mol. Biol. 12, 331-341.

_____ , 1967. The control of embryonic hemoglobin synthesis. In: Abercrombie, M. and Brachet, J. eds. , Adv Morphog. 6, 89-125. Academic Press, New York.

_____ , 1974. The beginnings of erythropoiesis in the yolk sac of the chick embryo. Ann. N.Y. Acad. Sci. 241, 39-47.

Yamada, K.M., Olden, K. and Pastan, I., 1978. Transformation sensitive surface protein: Isolation, characterization and role in cellular morphology and adhesion. Ann. N.Y. Acad. Sci. 312, 256-277.

_____, Yamada, S.S. and Pastan, I., 1976. Cell surface protein partially restores morphology, adhesiveness and contact inhibition of movement to transformed cells. Proc. Nat. Acad. Sci. U.S.A. 73, 1217-1221.

Yamada, T., 1937. Der Determinationszustand des Rumpfmesoderms im Molchkeim nach der Gastrulation. Wilhelm Roux' Arch. Entwicklungsmech. Org. 13, 151-270.
(Quoted by Muchmore, 1951; and Holtfreter and Hamburger, 1955).

_____ , 1940. Beeinflussung der Differenzierungsleistung des isolierten Mesoderms von Molchkeimen durch zugefügtes Chorda- und Neuralmaterial. Okajimas Folia Anat. JPN. 19, 131-197. (Quoted by Muchmore, 1951; Finnegan, 1953; and Holtfreter and Hamburger, 1955).

_____ , 1947. Extension of the potential theory of morphogenesis in amphibia (in Japanese). Zool. Mag. (Tokyo) 57, 124-126.
(reviewed in Yamada, 1950).

_____ , 1950. Dorsalization of the ventral marginal zone of the *Triturus* gastrula. I. Ammonia-treatment of the medioventral marginal zone. Biol. Bull. 98, 98-121.

Zagris, N., 1980. Erythroid cell differentiation in unincubated chick blastoderm in culture. J. Embryol. Exp. Morphol. 58, 209-216.

Zwilling, E., 1960. Some aspects of differentiation: Disaggregation and reaggregation of early chick embryos. Nat. Cancer Inst. Monogr. 2, 19-39.

_____ , 1968. Morphogenetic phases in development. Dev. Biol. Suppl. 2, 184-207.

Author Kessel J
Name of thesis Analysis of vascular Morphogenesis in the early chick embryo 1981

PUBLISHER:
University of the Witwatersrand, Johannesburg
©2013

LEGAL NOTICES:

Copyright Notice: All materials on the University of the Witwatersrand, Johannesburg Library website are protected by South African copyright law and may not be distributed, transmitted, displayed, or otherwise published in any format, without the prior written permission of the copyright owner.

Disclaimer and Terms of Use: Provided that you maintain all copyright and other notices contained therein, you may download material (one machine readable copy and one print copy per page) for your personal and/or educational non-commercial use only.

The University of the Witwatersrand, Johannesburg, is not responsible for any errors or omissions and excludes any and all liability for any errors in or omissions from the information on the Library website.