A COMPARATIVE STUDY OF ADRENAL GLAND DEVELOPMENT IN MOUSE AND CHICK EMBRYOS

BY

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DECLARATION

I hereby declare that this thesis is my own composition and that except where otherwise stated, the experimental work was performed by me alone.

None of the material in this thesis has been submitted for any other degree.

Taher A O Ba-Omar

DEDICATION

TO MY MOTHER

ACKNOWLEDGEMENTS

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CONTENTS

SUMMARY

In this work, I describe in detail the development of cellular patterns in mouse and chick adrenals, in an attempt to discover the morphogenetic mechanisms that produce these patterns.

Mouse adrenal gland tissues (chromaffin and cortical) are intermingled during embryonic life, but sort-out near the end of gestation into a central mass (medulla) of chromaffin tissue and a surrounding cortex. Once sorting out has occurred, the cells assemble into cortical zones and medullary cords.

Chick adrenal gland tissues (chromaffin and cortical) are intermingled with each other during embryonic and post-hatching life.

In mouse, the zona glomerulosa develops at day 1 and by day 4 both zona glomerulosa and fasciculata are obvious. By the end of the first week postnatal the cortex possesses three zones: zona glomerulosa, fasciculata and the developing X-zone. X-zone cells start to appear on the 4th day postnatal. The zona reticularis starts to develop while the X-zone is degenerating, about 32 days postnatal. An inner capsule is formed between the medulla and cortex of the male adrenal by 35 days postnatal (this process occurs later in females, during first pregnancy). Chromaffin cells start to be arranged in groups by 4 days postnatal and assume their final position in the centre of the gland (medulla) by 7 days postnatal. Different ..iation of chromaffin cells into A and NA cell types takes place in postnatal life.

In chick, light and dark cortical cells are seen from 15 days of incubation up to and including 19 days. Zonation of

cortical tissues is not seen. Differentiation of chromaffin cells into A and NA cell types takes place during post-hatching life. Sympathetic ganglion cells may contribute to the increased number of chromaffin cells by means of differentiation.

At TEM level the cell surface of mouse adrenal tissues is smooth with little sign of elongated cellular processes, throughout the period when sorting out of the tissue types is occurring. Some elongated cells are seen during sorting out stages, but not later. The cell surfaces of chick adrenal cells were similar, but were not studied during stages when significant cell sorting was occurring. No obvious changes occur with respect to cell junctions (between like and unlike cells) in all stages studied in both mouse and chick adrenals.

The distribution of fibronectin was analysed in embryonic mouse and chick by means of immunofluorescent labelling. Fibronectin was found in both mouse and chick embryonic adrenals at all stages studied, within the capsule, within blood vessels, around and between cortical cells, but only around groups of chromaffin cells, not within groups. There was no evidence for any quantitative variation in fibronectin that might have created an adhesive gradient to guide cell movement.

An unexpected results of this work was the discovery of cell death in both cortical and chromaffin tissues in embryonic adrenals of mouse and chick. These findings have not been reported before.

Morphometric analysis showed that mouse cortical tissue grows more rapidly than chromaffin during embryonic life and that mitosis is higher among corticals than chromaffins. It showed also that the medulla does not always form precisely in the centre of the gland.

In a final discussion chapter, I consider the role of cell death in the embryonic adrenal, and suggest this may be to maintain a loose enough structure to allow active cell sorting. This chapter also discusses the possible mechanisms of cell sorting and concludes that the most likely process to be involved is differential adhesion. Suggestions are also made for further work on adrenal morphogenesis.

PREFACE

I would like to introduce my work with some verses from the Holy Quran on the processes of embryogenesis which take place during development.

"Man We did create from a quintessence (of clay); Then We placed him as (a drop of) sperm in a place of rest, firmly fixed; Then We made the sperm into a clot of congealed blood; Then of that clot; We made a (foetus) lump; then We made out of that lump bones and clothed the bones with flesh; then We developed out of it another creature. So blessed be God, the best to create".

Holy Quran

Sura Mu-minun (Verses 12-14)

CHAPTER ONE

GENERAL INTRODUCTION

1.1 Historical Review

The adrenal glands are paired organs which are part of the endocrine system. They are ductless glands, which means that they secrete their products directly into the blood stream. They consist of two different tissues, the cortical (interrenal) tissue and the medullary (chromaffin) tissue.

The term adrenal or suprarenal usually implies a close anatomic and embryonic relationship with the kidneys. The adrenal tissues are present in all vertebrates, from cyclostomes to mammals, but they differ in their structural and functional arrangement.

The adrenal glands were first described by the anatomist Eustachius in 1563 and in 1805 Cuvier indicated that the mammalian adrenal glands possess two distinct regions, an inner and an outer region, which are known today as the medulla and the cortex respectively. Their functional significance was first described by Thomas Addison in 1849, whose name is now given to a disease which is related to a deterioration of the adrenal cortex. Arnold in 1866 indicated that the mammalian adrenal cortex is composed of three distinct zones. These are the zona glomerulosa (outer zone), zona fasciculata (middle zone) and zona reticularis (inner zone which borders the medulla). The study of adrenal gland development goes back to the last century. There were several workers in this field, including Gary (1852), Remak (1855), Kolliker (1861) and Balfour (1878). For reviews of this

early work, see Howard-Miller, 1927; Goldzieher, 1929; Waring, 1935; Romanoff, 1960 and Bethune, 1975.

1.2 Comparative Anatomy of the Adrenal Gland

1.2.1 Gross Anatomy (fig 1.1)

The gross anatomy of the adrenal gland is determined by the fate of the mesonephros. In mammals and birds the mesonephros degenerates, usually late in embryonic life, and the adrenocortical rudiments are left stranded, anterior to the adult metanephric kidneys (Chester Jones, 1976). In fish and amphibians mesonephric kidneys are retained in adulthood, and therefore the adrenocortical tissues retain their relative embryonic position, on the ventral side of the kidney (Chester Jones, 1976).

The mammalian adrenal glands are discrete encapsulated organs which are located on the anterior pole of the kidneys though they vary in their closeness to the kidneys and to the main abdominal blood vessels. They take various shapes, spheroid, oval, elliptical, cylindrical or rod like depending on the species. Generally the mammalian adrenal gland consists of two distinct regions, the cortex - outer region, and the medulla - inner region, except in the sea lion (Gorbman & Bern, 1962 and Turner & Bagnara, 1971) where the arrangement of the two tissues resembles that of the avian adrenal gland (discussed later). The cortex consists of three zones: these are the zona glomerulosa, zona fasciculata and zona reticularis from outer to inner respectively. The medulla is made up of so-called chromaffin cells, named from their yellow-brown staining with chromium salts.

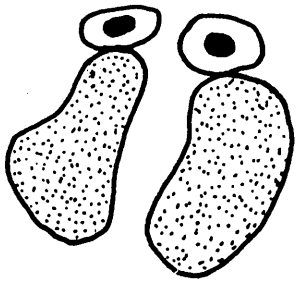
The avian adrenal glands are also discrete encapsulated organs. They lie at the anterior end of the kidneys on each side

Fig. 1.1. Comparative anatomy of the adrenal (suprarenal) gland of the vertebrates. The kidney is stippled, the adrenal cortical tissues are clear and the adrenal medullary tissues are dark. Redrawn with modification from D. O. Norris. Vertebrate Endocrinology, Lea and Febiger. Philadelphia, 1985.

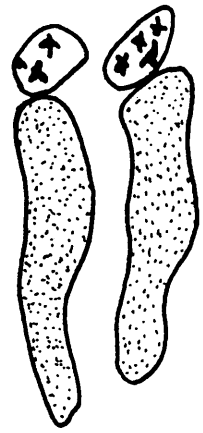
a-d=adrenal gland is located on the anterior side of the kidney.

e-f=adrenal gland is located on the ventral side of the kidney.

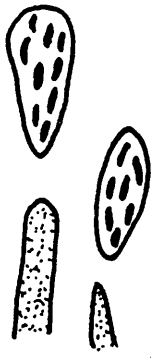
Fig. 1.1



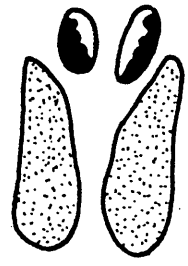
a. Mammals



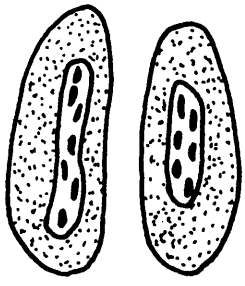
b. Birds



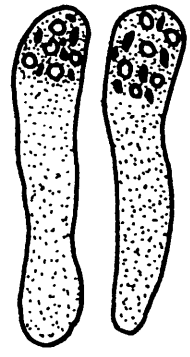
c. Reptiles (snakes)



d. Reptiles (lizards)



e. Amphibian (Anura)



f. Fish (Teleostei)

of the aorta and the inferior vena cava. In most birds, the adrenals are wholly or partly covered by the gonads, especially the left adrenal gland. Each gland consists of two different tissues, the cortical (interrenal) and the medullary (chromaffin) tissues. The two tissues are intermingled with each other. The "cortical" tissue is arranged in solid cords, whereas the chromaffin tissue is arranged in clusters or in small group of cells. There is no cortico-medullary arrangement. Although this arrangement is lacking in birds, I will for convenience continue to call the avian equivalent of the mammalian cortex, cortical cells.

The reptilian adrenal glands resemble the avian ones: that is, the two tissues are intermingled. The adrenal glands are somewhat elongated in shape and set on the anterior side of the kidneys and are seen associated with the main blood vessels and often adjoining the gonads. In some lizards and snakes, the chromaffin tissue forms a distinct band at the periphery of the gland, partly surrounding the central mass of the cortical tissue, almost the opposite of the mammalian arrangement (Gorbman & Bern, 1962 and Turner & Bagnara, 1971).

The amphibian adrenal glands are elongated organs found on the ventral surface of the mesonephros. The arrangement of the two tissues differs from one species to another: some species possess the two tissues in the same organ. On the other hand, in some species, the two tissues are separate. The "cortical" tissues are associated with the kidneys, whereas the chromaffin tissues are associated with the abdominal blood vessels.

In fish, adrenal gland components never form a discrete organ, but rather form separate structures. The "cortical" tissues are associated with the kidneys, lying on the anterior

end or between the kidneys, whereas the chromaffin tissues are usually associated with the abdominal blood vessels.

1.2.2 Blood and Nerve Supply

The adrenal gland is one of the most vascular organs in the body. It gets blood from all the main arteries which pass near such as the dorsal aorta, renal arteries, phrenic arteries and coeliac trunk, all of which may give branches that supply the adrenal gland depending on species. The adrenal gland has a well developed venous drainage and all venules empty into the central vein of the gland which drains the blood, either into the renal vein which drains into the vena cava, or directly into the vena cava.

The adrenal gland is innervated by nerves which originate in the spinal cords such as the phrenic or splanchnic nerves, renal or celiac plexuses, which send branches to the adrenal gland depending on species. Coupland (1965b) showed that both myelinated and non-myelinated nerve fibres of the pre-ganglionic sympathetic nervous system innervate the adrenal medulla of the rat. This is also confirmed by the work of Grynszpan-Winograd (1974) on the hamster.

1.3 Functions of the Adrenal Gland

The adrenal glands have a vital function in controlling and maintaining the essential homoeostatic mechanisms of the body. Their functions are vital to life and growth even in early development. They secrete steroid hormones (cortical tissue) and catecholamines (chromaffin tissue). The steroid hormones (more than 50 hormones are found to be synthesised in mammalian cortical tissue) have an influence on carbohydrate and protein

metabolism and also have anti-inflammatory effects and regulate electrolyte relationships, especially Na+ and K+. In contrast, the catecholamines (Adrenaline -A- and Noradrenaline -NA) are stress hormones which help to regulate involuntary functions of the body such as heart rate, blood vessel diameter, intestinal movement and the dilation of the pupil of the eye. These effects prepare the body for various conditions of stress, including pain, fear and muscular activity. High levels of catecholamines are present in the fetus during delivery and at birth, to regulate breathing and to break down the stored energy into forms that can nourish cells once the umblical cord is cut (Lagercrantz & Slatkin, 1986).

1.4 Development of the Adrenal Gland in Mammals and Birds (fig 1.2)

The adrenal glands of mammals and birds share the same embryonic origin and function. They show similarities in their development to a certain stage of development, but will differ in their later development resulting in the differing morphologies of the adult glands.

The adrenals arise by the association of two distinct embryological elements, the cortical and the medullary elements. The two elements are embryologically, anatomically and histologically unrelated. They are derived from two different tissues, which unite together in embryonic life to form a single organ. The cortical element is the first to appear followed by the medullary element which migrates into place later.

1.4.1 Development of the Adrenal Cortical Element

The coelomic epithelium of the trunk region contributes to the formation and the development of the adrenal cortical tissue. The underlying mesenchyme may contribute a little to the developing adrenal cortex but mainly to the adrenal gland's capsule (Lever, 1955). Therefore, the adrenal cortex has a mesodermal origin.

1.4.1.1 The Mammalian Adrenal Cortical Element

The cortical anlage usually buds off from the roof of the peritoneum between the aorta and the urinogenital ridge. In the mouse embryo, the initial appearance of the cortical anlage was observed on about the 11th day of embryonic life by Waring (1935), Fernholm (1971) and Theiler & Müntener (1974).

In the mouse embryo, the cortical anlage is consolidated and separated from the general mesenchyme by a sheath on about the 13th day of embryonic life (Waring, 1935). The whole gland is surrounded by a capsule and can be dissected out. By this time vascularisation is already established (Waring, 1935).

Lever (1955) studied the development of the adrenal cortex of the rat, and showed that the cortical anlage appears on about the 12th day of embryonic life. In the rabbit, the initial appearance of the cortical anlage was observed on the 12th day of embryonic life by Joseph *et al* (1973) and on about the 14th day of embryonic life by Coupland & Weakley (1968). In the human it was seen on about the 4-5th week of embryonic life (Panskey, 1982).

The process of growth and differentiation of the adrenal cortex starts during embryonic life and may go on for several weeks after birth, depending on species. As a result of this,

Fig. 1.2a. A schematic drawing of trunk region showing the position of the developing human embryo adrenal gland redrawn with modification from J. Langman. Medical Embryology, 4th. ed. Willaims & Wilkins, Baltimore/London. 1981.

Fig. 1.2b. Schematic drawings showing the developing adrenal gland of the chick embryos, also their position with respect to the urinogenital system at three different stages of development. Redrawn with modification from A. L. Romanoff. The Avian Embryo. Macmillan Co. N.Y. 1960.

A=developing adrenal gland, Ar=aorta, D=dorsal root ganglion, dce=day chick embryo, G=gut, Gr=urinogenital ridge, M=mesonephros, Mt=metanephros, N=notochord, Nt=neural tube O=ovary, P=parasympathetic ganglion, S=sympathetic ganglion

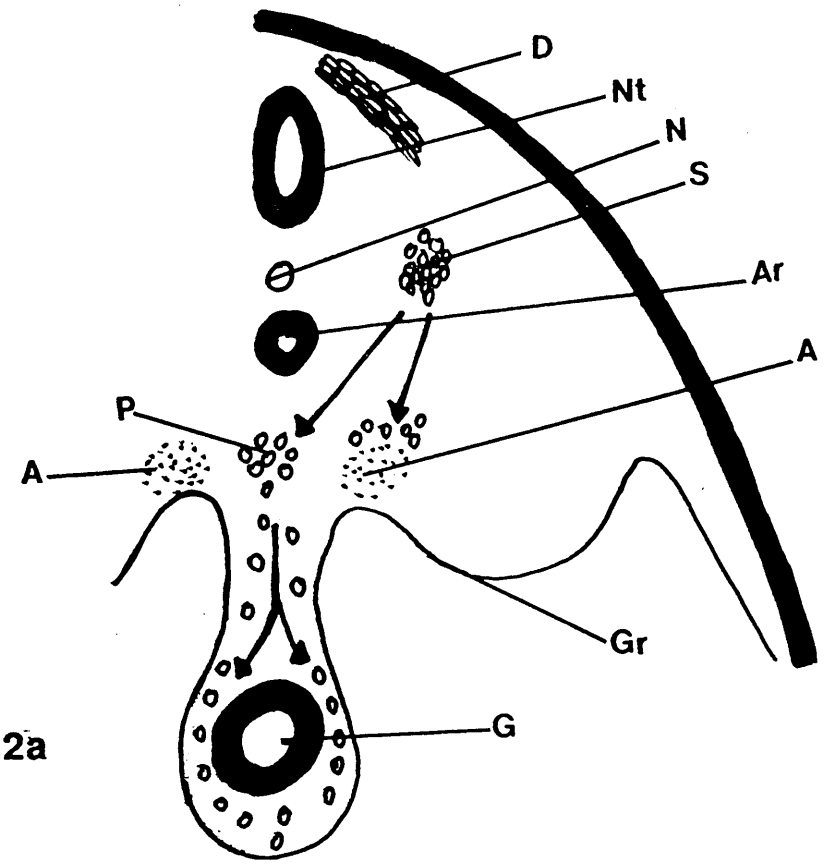


Fig. 1.2a

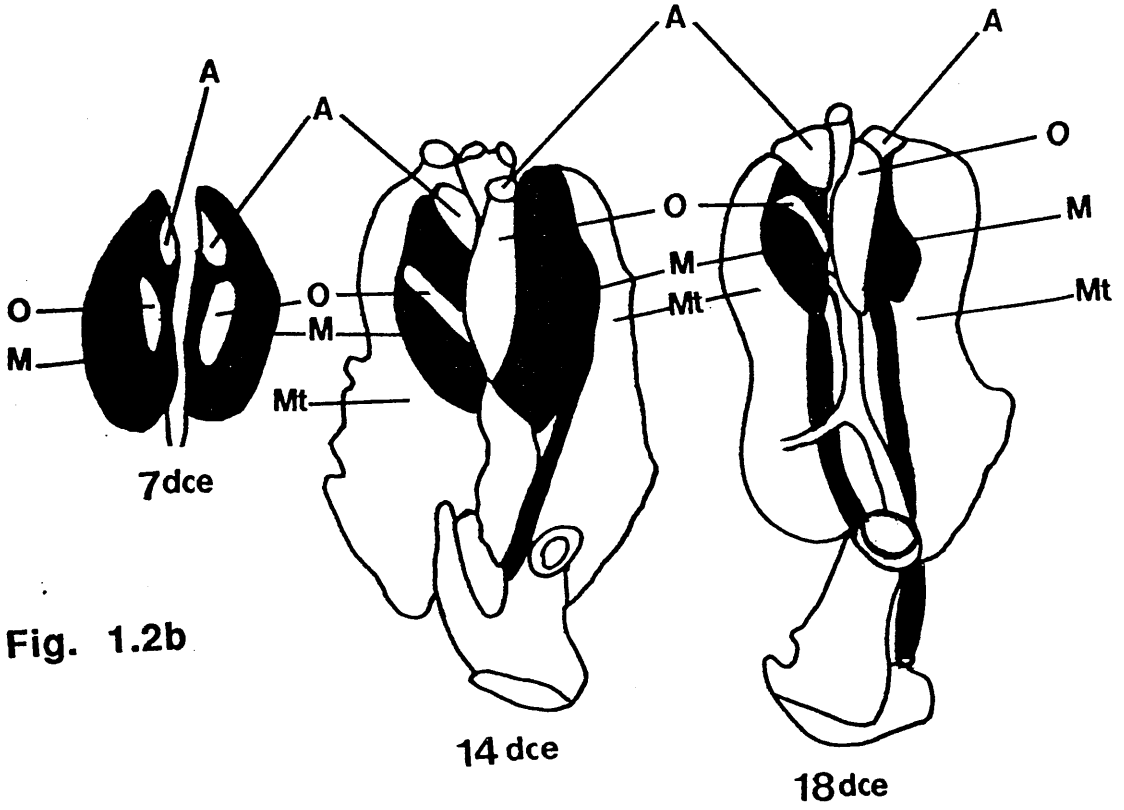


Fig. 1.2b

cortical cells will be arranged in three zones as follows: zona glomerulosa -outer zone-, zona fasciculata -intermediate- and finally zona reticularis -innermost zone bordering the medulla. Some of these zones may be developed in late embryonic life and the early days after birth, especially the zona glomerulosa and zona fasciculata, whereas the zona reticularis will usually develop later.

1.4.1.2 The Chick Adrenal Cortical Element

The initial appearance of the chick adrenal cortical anlage was seen as early as the 4th day of incubation (Dawson, 1953; Romanoff, 1960; Sivaram, 1965 and Mezhnin, 1969). The anlage is located ventral and medial to the mesonephros, ventral to the abdominal aorta and dorsal to the hind gut. As soon as formation takes place, the cortical tissue leaves the epithelium and moves dorsally (Romanoff, 1960). The anlage will take its final position between the aorta and the mesonephros about the 6th day of incubation and start to separate itself from the mesonephros by strands of mesenchyme (Sivaram, 1965). The cortical tissues will start to arrange themselves into cords about the 7th day of incubation and by the 8th day they have well formed cords (Dawson, 1953; Romanoff, 1960 and Sivaram, 1965). About the 8th day, the capsule starts to grow and vascularisation begins, shown by the presence of blood cells between the cortical cords (Romanoff, 1960 and Sivaram, 1965).

1.4.2 The Medullary Elements

It is now known that the medullary elements have a neuro-ectodermal (neural crest) origin (Hammond & Yntema, 1947; Weston, 1963; Fernholm, 1971 and Le Douarin & Teillet, 1974). It is the

sympathetic nervous system of the trunk region which contributes to the formation of the adrenal medullary elements.

There had long been suspicions about the adrenal medullary elements origin since the study of the development of the adrenal gland began back in the last century. It was originally believed that the medullary elements have a mesodermal origin (for review see Waring, 1935). Rau & Johnson (1923) and Willier (1930) put forward evidence that the medullary elements of the adrenal gland are derived from the sympathetic nervous system. The first reasonably conclusive demonstration of this was by Hammond & Yntema (1947). This hypothesis was confirmed later by Weston (1963); Fernholm (1971) and Le Douarin & Teillet (1974) using modern techniques (auto-radiography and the quail nucleolar marker).

The sympathetic nervous system is derived from neural crest cells. The migrating neural crest cells will be localised lateral and ventral to the notochord in the area around the aorta. It is the latter which will contribute to the formation of the adrenal gland medullary elements and the aortic plexi (Hammond & Yntema, 1947; Fernholm, 1971 and Le Douarin & Teillet, 1974). The ventral stream of the migrating neural crest between the neural tube and the somites of the trunk region gives rise to the sympathetic nervous system of the trunk.

1.4.2.1 The Mammalian Adrenal Medullary Element

The presumptive medullary cells form an aggregate on the outside of the cortical anlage. Medullary cells then migrate into the cortical anlage as individuals and as small groups (Waring, 1935 in the mouse and Lever, 1955 in the rat).

Fernholm (1971) used auto-radiography to determine the migration pattern of the sympathetic tissues in the mouse embryo, and showed that it is the caudal part of the thoracic and the cranial part of the lumbar regions which give rise to the medullary elements. In the mouse, the appearance of the medullary element in the cortical anlage was first observed on about the 11-12th day of embryonic life (Theiler & Müntener, 1974). On the other hand, Waring (1935) and Fernholm (1971) observed the first migration of medullary cells into the cortical anlage on the 13th day of embryonic life. They all agree on the day that migration into the gland ceases, which is the 14th day of embryonic life.

In the rat embryo, the initial appearance of the medullary elements in the cortical anlage was observed on the 15th day by Millar & Unsicker (1981), whereas Lever (1955) indicated that it starts on the 16th day. Coupland & Weakley (1968), studying the development of the chromaffin tissues in the rabbit, indicated that the initial appearance of the medullary elements in the cortical anlage is seen on the 16th day. In the human, the first sign of migration of the medullary elements into the cortical anlage is at about the 6-7th week of fetal life (Panskey, 1982).

Chromaffin tissues are not confined to the adrenal gland, but are also found associated with other organs such as the abdominal blood vessels, especially the aorta. They are called para-aortic bodies by Coupland (1954, 1960). They are found at the beginning of the superior and inferior mesenteric arteries of man, mouse and guinea-pig (Coupland, 1954, 1960), in rabbit (Coupland & Weakley, 1968) and in opossum (Spagnali *et al*, 1987). They actively proliferate during embryonic life and after birth,

but they start to degenerate later on in early life, especially in man and mouse, but they persist throughout life in guinea-pigs (Coupland, 1960). They have the same origin as that of the chromaffin cells of the adrenal gland.

1.4.2.2 Chick Adrenal Medullary Element

The medullary (chromaffin) tissues of the chick, have a similar origin to those of the mouse. The sympatho-chromaffin tissues (undifferentiated chromaffin cells) form an anlage adjacent to the developing adrenal cortical anlage. Dawson (1953) used silver impregnation (it blackens the chromaffin cells) to detect the early formation of the medullary tissue. The initial migration of the sympatho-chromaffin cells into the cortical anlage is seen on the 5-6th day of incubation (Dawson, 1953 and Sivaram, 1965). Sivaram (1965) indicated that active penetration of the undifferentiated medullary cells occurs from all directions. By the 9th day of incubation, most chromaffin cells have entered the cortical anlage (Romanoff, 1960 and Sivaram, 1965). Two sympathetic nerve trunks, primary and secondary, occur in bird embryos (Rau & Johnson, 1923 and Willier, 1930). The primary sympathetic trunk develops on about the 4th day of incubation and reaches its maximum on about the 5th day after which it gradually disappears (Willier, 1930). The secondary or the definitive sympathetic trunk starts to develop on about the 6th day. Rau & Johnson (1923) indicated that the primary sympathetic trunk may be incorporated in part at least in the secondary sympathetic trunk.

Although the primary sympathetic trunk makes the major contribution to the formation of the medullary elements, Willier (1930) and Dawson (1953) showed that the secondary sympathetic

trunk may also make a supplementary contribution to the formation of the medullary elements of the adrenal gland.

1.4.3 The Development of a Single Gland

The development and growth of the adrenal gland as a single organ begins as soon as the migration and the penetration of the medullary cells into the cortical anlage takes place. It also involves the formation of the gland's capsule, the development of vascularisation and innervation. The differentiation and maturation of the cortical and medullary tissues takes place partly during embryonic life and partly after birth.

In the following chapters, further details will be discussed with respect to the development of the adrenal gland of mouse and chick embryos.

1.5 The Aims of This Study

The aim of this project is to make a comparative study on the development of the adrenal gland of mouse and chick embryos concentrating on morphogenetic processes. The study is not concerned with the early formation of the two separate anlagen, but rather the gland as a single organ.

This study was carried out to investigate some of the unknown features about the adrenal gland's development, such as what attracts the neural crest cell derivatives (chromaffin cells) to move towards the adreno-cortical anlage and migrate into the centre (mouse). What sort of movement, is it active or passive? How do chromaffin cells know where to go and when to stop their movement? The internal structures of adrenal glands of mammals show different arrangements to those of birds: chromaffin

cells are arranged in a mass in the centre of the gland in mammals, but in birds they are arranged in patches, irregularly scattered throughout the gland. Why are they arranged in different patterns; is it to do with properties of the chromaffin or the cortical tissues or both?

In recent years there has been considerable interest in the way migrating cells find their ways to their final and correct positions during development. The fact that we have two main cell types sorting out from one another also makes this an interesting system to study. To help to answer some of these questions, this study was carried out in the following ways:

In the mouse, the study includes the morphology of the embryonic and postnatal glands up to the 35th day after birth, using light, transmission and scanning electron microscopy to look for any changes that may occur during development. Tissue culture was used in this study to look at the behaviour and the interaction of both cortical and medullary cells in an artificial environment. A quantitative analysis of both cortical and medullary tissues was carried out to find out the relative volumes of both tissues and the relative position of the medulla as development progresses.

In the chick, light, transmission and scanning electron microscopy was carried out on embryonic and post-hatching stages.

Distribution of fibronectin was also studied in both mouse and chick embryo adrenal glands.

Some unexpected features emerged in this study, notably the role of cell death in adrenal development in the mouse and chick.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Animals

The practical work of this project was carried out on mice and chick adrenal glands. The animals and the eggs were obtained from the Laboratory of the Developmental Biology Building, Department of Zoology, Glasgow University.

2.1.1 Mice

The Glasgow hybrid breed (GH) (stock obtained by crossing four inbred strains of mice A2G/Tb, A/Tb, C57BL/Tb and GFF, Barnett & Little, 1968 and Barnett & Munro, 1970) mouse population was used in this study. The animals were maintained under standard conditions of temperature (20-22° C) and lighting (9 hr of light and 15 hr of darkness). Food and water were available *ad libitum*. Females' oestrus cycles were brought about by exposing females to some of the bedding materials from the males' cages. Males were usually put within the females' cages - for mating- in late afternoons or early evenings. Females were then examined every following morning for vaginal plugs. The day a positive plug was obtained was designated as day 0 of pregnancy.

2.1.1.1 The Adrenal Gland of the Mouse Embryo

Pregnant females (14-18th day of pregnancy and some of the 13th day) were sacrificed by cervical dislocation. The abdomen was swabbed with 70% alcohol prior to the performation of

laparotomy. The uterine horns were removed using scissors and fine forceps and placed in a petri dish containing Tyrode's balanced salt solution. The embryos were decapitated as soon as they were dissected out and then transferred into fresh Tyrode's solution. The adrenal glands were dissected out using a Wild M5 binocular dissecting microscope with X10 eyepiece and X6 or X10 or X25 objective magnification, depending on the age of the embryo. Prior to the 14th day of embryonic life, it was not possible to dissect out the adrenal glands as a discrete entity, and therefore the 13 day specimens were removed *en bloc* with the surrounding tissues. The adrenals were then transferred into fresh Tyrode's solution, where they were freed from adhering tissues and fat. The adrenals were then processed as required for different experiments (see section 2.2).

2.1.1.2 The Adrenal Gland of the Postnatal Mouse

Newly born mice of age 1-10 days and 14, 21, 32, 35 days were also used in this study. Chloroform was used as a killing agent. The mice were decapitated and laparotomy was performed on each mouse using fine scissors and forceps. The adrenals were dissected out, using a Wild M5 binocular dissecting microscope, and then transferred to Tyrode's solution, where they were freed from adhering tissues and fat. Further treatment is detailed in section 2.2.

2.1.2 The Adrenal Gland of the Chick Embryo and the Post-Hatching Chick

White leghorn eggs were incubated at 37-38°C. The eggs were opened at the blunt end when they reached the desired day of development, that is between the 15th and 19th day of incubation.

The embryos were decapitated as soon as the eggs were opened using a pair of scissors. The decapitated chick embryos were placed in Tyrode's solution. The trunk region of each embryo was opened and the adrenal gland was dissected out using a Wild M5 binocular dissecting microscope. The glands were placed in fresh Tyrode's solution where they were freed from adhering tissues and organs.

Post-hatching chicks were sacrificed using chloroform as the killing agent. The adrenal glands of 2 day and 10 days old chicks were dissected out and then freed from adhering tissues and organs. Further treatment is detailed in section 2.2.

2.2 Processing of the Tissues for Microscopy

The main aim of this study was to look at cell interactions in adrenal glands. This includes cell junctions, and detailed changes in cell shape during the migration of the chromaffin tissues to form either the medulla as in mammals or patches as in birds. Therefore it was necessary to use the same fixation procedures for light and electron microscopy, so that the two levels of resolution could be most reliably compared. The combination of half strength Karnovsky (Karnovsky, 1965) as a fixative and araldite resin as an embedding agent were used routinely for this work. Other fixatives such as Bouins, chromaffin reaction and formalin as fixatives and paraffin wax as an embedding agent were also used for light microscopy only.

For some of the experiments, other fixative procedures were used (see later).

I also tried a number of other procedures (see section 2.5.4) for distinguishing chromaffin and cortical cells,

especially in tissue culture experiments, but without useful results.

2.2.1 Fixation of the Tissues

The adrenal glands of both mice and chicks were processed as follows for araldite embedding:

1. Adrenals were fixed in ice-cold half strength Karnovsky for 1-2 hr (mouse) and 2-3 hr (chick)
2. Wash with cacodylate buffer 3X5 min
3. Post fixed in osmium tetroxide 1-2 hr (in fume cupboard)
4. Wash with cacodylate buffer 3X5 min (in fume cupboard)
5. Dehydrated in 70% alcohol 10 min
6. Dehydrated in 90% alcohol 10 min
7. Dehydrated in absolute alcohol 3X10 min
8. Dehydrated in absolute alcohol/propylene oxide (3:1) 10 min
9. Dehydrated in absolute alcohol/propylene oxide (1:1) 10 min
10. Dehydrated in absolute alcohol/propylene oxide (1:3) 10 min
11. Dehydrated in propylene oxide 3X10 min
12. Infiltrated in a mixture of propylene oxide/araldite resin (1:1) for 24 hr on a rotator.
13. Embedded in pure araldite resin in foil trays and then placed in an oven at 60°C for 24-36 hr, until polymerisation occurred.

    Some specimens were fixed in Bouins for paraffin wax embedding, for routine staining.

2.2.2 Sectioning, Staining and Examination of Tissues

2.2.2.1 Light Microscopy (LM)

Semithin araldite (sections 1 micrometer (μm) approximately) were cut using glass knives on a Reichert OMU3 ultramicrotome.

The sections were arranged in serial order, in groups of 6-8 sections, mounted on glass slides and then stained with toluidine blue in 1% borax or ammoniacal silver carbonate (Tramezzoni *et al*, 1964).

Wax sections (5-6 μm) were cut on a Beck microtome and then stained with haemotoxylin and eosin (H&E).

All slides were mounted with DPX and then examined with a Leitz SM Lux light microscope. Photographs were taken using Kodak Panatonic-X on a Wild M20 photomicroscope.

2.2.2.2 Transmission Electron Microscopy (TEM)

Ultrathin sections of adrenal gland tissues were cut with glass knives on a Reichert OMU3 ultramicrotome. Sections exhibiting silver to gold interference colours were collected and mounted on uncoated 300 G copper grids. They were stained with uranyl acetate and lead citrate (Reynolds, 1963) for 5 min each with water and NaOH washes between each stain. The stained sections were examined under initially a AEI 801 and latterly a Zeiss 902 Transmission Electron Microscope.

2.2.2.3 Scanning Electron Microscopy (SEM)

The above procedure for section 2.2.1 was followed up to step (4). The adrenals were then dehydrated in acetone instead of alcohol as follows:

5.	Dehydrated in 70% acetone	10 min
6.	Dehydrated in 90% acetone	10 min
7.	Dehydrated in absolute acetone	2X10 min
8.	Dehydrated in dry absolute acetone	10 min

9. The adrenals were dried in the critical point dryer for 1hr.

10. The adrenals were mounted on small aluminium stubs, using colloidal silver.
11. They were coated with gold/palladium in a sputter coater for 6 min.

The adrenal glands were examined under a Philips SEM 500.

Specimens were fractured to expose internal structure, either before fixation, or after fixation, or while in acetone just before drying or after they were already dried.

2.3 Quantitative Measurement of the Mouse Adrenal Glands

Morphometric analysis of mouse embryonic and postnatal adrenal glands was carried out using serial semithin araldite sections, as follows. One section of each group was drawn on a white sheet of paper size A4. The outline of the adrenal gland structures, that is the capsule-cortex boundary and cortex-medulla boundary, were traced with pencil with the aid of a camera lucida attached to a Wild M20 light microscope using X10 eyepiece and an objective magnification of X10. A scale of 0.5 mm was drawn out as a standard for the drawings at the end of each page. The drawings were then magnified to 150% using a Canon NP-4035 Af copier machine onto paper size A3, so that the smallest drawings could be accurately traced with a computer tracing cursor. The capsule-cortex boundary was traced first and then the cortex-medulla boundary with a four-button cursor on a Cherry A3 digitising tablet.

A computer program designed to calculate the area, perimeter and volume was used on a BBC Master microcomputer (designed by Dr. M. D. Burns, Zoology Department, Glasgow University). It was designed to do the following:

A. Calculate the area, perimeter and volume of the outer

structure (cortex).

B. Calculate the area, perimeter and volume of the inner structure, medulla, containing some cortical tissue.

C. Calculate the area, perimeter and volume of the cortical tissue still present within the medullary region.

The net area and volume of each structure of 15 and 17 days embryo and 7 day postnatal adrenal were calculated as follow:

Cortical tissue=A+C

Chromaffin tissue=B-C

2.4 Immunofluorescence

Distribution of fibronectin (FN) in developing adrenals was determined on mice embryos (14, 16 and 18 day embryonic life) and chick embryos (15, 17 and 19th day of incubation). The adrenal glands were dissected out as previously described in sections 2.1.1.1 and 2.1.2. Two fixatives were used, 96% ethanol containing 1% acetic acid (Harrisson *et al*, 1984) and 4% paraformaldehyde (Armstrong & Armstrong, 1984). Better results were obtained by using the 96% ethanol fixative. Therefore the results which are described in chapter 5 are entirely from this method.

The following procedure was carried out for fixation and staining of the adrenal glands.

2.4.1 Fixation

1.	Fixed in 96% ethanol with 1% acetic acid	4-5 hr
2.	Dehydrated in 95% alcohol	10 min
3.	Dehydrated in absolute alcohol	10 min

4. Cleared in xylene (check for transparency) 5-10 min
5. Infiltrated with xylene/paraffin wax (1:1) 15 min
6. Infiltrated with paraffin wax 1 15-20 min
7. Infiltrated with paraffin wax 2 15-20 min
8. Embedded and blocked in pure paraffin wax.

The blocks were sectioned at 6 micrometers (μm) on a Beck microtome. The sections were placed on standard glass microscope slides which had been previously coated with albumin (10-15 sections per slide).

2.4.2 Dewaxing and Rehydration

1. Histoclear 6 min
2. Absolute alcohol 6 min
3. 95% alcohol 3 min
4. 90% alcohol 3 min
5. 70% alcohol 3 min
6. 50% alcohol 3 min
7. 30% alcohol 3 min
8. Rinse and wash with distilled water

2.4.3 Staining

1. Washed in phosphate buffer saline (PBS) 15-20 min
2. Incubated in anti-fibronectin antibody diluted in PBS (1:60) for 40-50 min at room temperature (the antibody was human anti-fibronectin, raised in rabbit (Dakopatts) and was a gift kindly given by Dr. C. Skerrow, Dermatology Department, Glasgow University).
3. Rinsed and then washed in PBS 5 min
4. Incubated in fluorescein isothyocyanate (FITC) labelled anti-rabbit IgG anti-serum diluted in PBS (1:200) for 40-50 min

at room temperature (FITC from Protozoology Unit, Zoology Department, Glasgow University).

5. Rinsed and washed in PBS 5 min

6. Mounted with 50% (W/V) glycerol/PBS.

The control slides were incubated in normal rabbit serum (NRS) or in PBS for 20-30 min instead of the anti-fibronectin and the rest of the procedure was as described above.

The stained sections were examined with a Leitz Ortholux 11 microscope using incident light fluorescence with an HB 050 light-pressure mercury vapour lamp. For the FITC reaction, 2X KP490 (exciting), KT510 (dichroic mirror) and K515 (suppressing) filters were used. Colour photographs were taken using Kodak Ektachrome 400 ASA.

2.5 Tissue Culture

2.5.1 Preparation of Tissues

All the work with the culture experiments was performed under sterile conditions. Adrenal glands of mouse embryos (15-18th day) and some of postnatal (up to 1 week) mice were used in this study. The pregnant females were sacrificed by cervical dislocation and quickly swabbed with 70% alcohol. Laparotomy was performed in a sterile cabinet and the uterine horns were removed and transferred into sterile Tyrode's solution in sterile glass petri dishes. The embryos were dissected out from the uterus and transferred into fresh sterile Tyrode's solution, where they were decapitated. Each embryo was dissected out for its adrenal glands using a Wild M5 binocular dissecting microscope, in an empty sterile petri dish to avoid contamination. The adrenal glands

were removed and quickly transferred into culture medium (see below) in a small sterile (35 mm) Falcon plastic dish.

The postnatal mice were killed by means of chloroform in a desiccator. They were then swabbed with 70% alcohol before being decapitated. Laparotomy was performed on each mouse and the adrenal glands were dissected out and quickly transferred into medium in a small sterile (35 mm) Falcon plastic dish.

The adrenal glands from both pre- and postnatal mice were freed from adhering tissues and fat. They were cut into small pieces or into two halves or left complete, according to the experimental requirement.

2.5.2 Medium

A defined medium consisting of Hams F12 (90 ml) with L-Glutamine (Gibco-Scotland), fetal calf serum (FCS) (10 ml), penicillin (2ml) and streptomycin (2 ml) both by Glaxo-England was used. The components were mixed together fresh under sterile conditions before being used.

2.5.3 Methods of Culturing

All cultures were usually set in the afternoon and were examined every morning using a Wild M20 inverted microscope equipped with phase contrast optics. Cultures were maintained in a humid atmosphere at 37°C in a National CO_2/Air incubator. In some cases, medium was changed at intervals of 2-3 days.

2.5.3.1 Hanging Drop Method

Small pieces of adrenal glands were placed on 32x32 mm coverslips with a few drops of medium. Excess medium was pipetted off with a 0.7 mm pipette. The coverslips were sealed with wax on

to glass cavity slides. The slides were placed in the incubator and cultured for 2-4 days.

2.5.3.2 Glass Coverslips Method

Small pieces of adrenal glands were placed on small glass coverslips (15 mm) with a minimum amount of medium. The coverslips were placed in Falcon plastic dishes (35 mm) with 2-3 coverslips in each dish. The excess medium was pipetted off with a 0.7 mm pipette to allow the tissue to stick to the coverslip. The dishes were then placed in a large covered plastic box, containing cotton wool on its sides soaked in sterile Tyrode to ensure the explants were kept in a humid atmosphere, and placed in the incubator at 37°C with air/CO_2 (95/5%) atmosphere and then left overnight. Extra medium was usually added the following morning. Fresh medium was supplied every 2-3 days. The tissues were cultured for 1-15 days. Some of these cultures were used for time lapse cine micrography, others for SEM and TEM.

2.5.3.3 Falcon Plastic Dishes (35 mm) Method

In one set of experiments it was necessary that cultured adrenals should not attach to the culture dishes. Complete glands, or pieces of gland, were placed in Falcon plastic dishes about half filled with medium. Detailed procedure will be given in Chapter 6.

2.5.4 Time-Lapse Cinemicrography

Time lapse cinemicrography of cultures was carried out in a warm room set at 37°C using a Wild M40 inverted microscope and a Bollix camera, with Kodachrome 40, 16mm colour film. Cultures

were photographed at intervals of 60-90 seconds/frame using a X10 phase contrast objective.

2.5.5 Fixation and Staining of Cultures

Some of the hanging drop and coverslip cultures were fixed in half strength Karnovsky (some were processed for LM and TEM, whereas others were processed for SEM), glutaraldehyde, formalin or Bouin. Some cultures were stained with toluidine blue, sudan black B, ammoniacal silver carbonate and haemotoxylin and eosin (H&E).

The purpose of using different fixatives and different stains was to distinguish and identify the two main cell types of the adrenal gland.

CHAPTER THREE

Development of the Mouse Adrenal Gland

3.1 Introduction

3.1.1 Mammalian Adult Adrenal Gland Structure

The adult mammalian adrenal glands are paired organs. They lie on the anterior side of the kidneys, one on each side. They are generally encased in a thick capsule of connective tissue, through which blood and nerve supplies penetrate. The adrenal gland consists of two distinct tissues, which are arranged into two regions; an outer region, "the cortex" which consists of cortical tissue, and a central region, "the medulla" which consists of chromaffin tissue. The cortex is composed of three zones (layers)- anatomically and histologically different. The cortical zones can be identified on the basis of their appearance and the distributions of various cytoplasmic organelles within the cortical cells. Those cortical zones are arranged as follows:

(1). The zona glomerulosa is the outermost zone located just beneath the capsule of the gland. It consists of small cells which are arranged in groups or cords, separated from each other by a thin connective tissue layer and blood sinusoids. The cells of this zone are poor in lipid droplets and are moderately secretory.

(2). The zona fasciculata is the intermediate zone, located under the zona glomerulosa. It consists of larger cells which are arranged in long fascicles (1-2 cells thick). Blood sinusoids are present between the fascicles. The cells of this zone are rich in lipid droplets, hence their cytoplasm

looks vacuolated with a "spongy" appearance after tissue processing. They are the most secretory cells of the cortex. The zona fasciculata forms the largest portion of the whole cortex.

(3). The zona reticularis is the innermost zone, located between the zona fasciculata and the medulla of the adrenal gland. It consists of small cells, arranged in anastomosing cords. This zone is the least secretory and is poor in lipid droplets.

Lever (1955) in the rat, and Bloodworth & Powers (1968) in the dog, showed that a fourth zone is morphologically distinct between the zona glomerulosa and zona fasciculata. They called this the intermediate zone. Lever (1955) claimed that this zone is a lipid free zone.

The medulla consists of the chromaffin cells. It is surrounded by the zona reticularis of the adrenal cortex. The chromaffin cells are round or polygonal in shape and arranged in groups separated by thin layers of connective tissue and blood sinusoids. The medulla has two cell types, adrenaline (A) cells and noradrenaline (NA) cells. Both cell types are randomly dispersed throughout the medulla. The two cell types can be identified on the basis of their secretion and their affinity to stain.

There have been reports in some mammals of an inner capsule, separating cortex from medulla. This has been described in the mouse adrenal gland (Waring, 1935; Holmes & Dickson, 1971; Hirokawa & Ishikawa, 1974 and Deacon *et al*, 1986). Bloodworth & Power (1968) in the dog found that the junction between cortex and medulla is made up of thick bands of collagen fibres, though these fibres are not continuous, and concluded that this

structure is not a true capsule.

There have been numerous reports on size and functional differences in adrenal glands. Variation in adrenal weight depends on the animal's strain, its environment and its sex. Rogers & Richter (1948) showed that wild Norway rats have heavier adrenals than domesticated ones. Hartman *et al* (1931) reported that when normal rats were exposed to the cold, their adrenal glands became larger. The sex of the animal has an affect on the size of the adrenal glands. Most workers have shown that the female's adrenal glands are heavier than the male's: for example, Shire (1965) and Badr *et al* (1968) in the mouse; Rogers & Richter (1948); Malendowicz (1974a, b); Majchrzak & Malendowicz (1983); Malendowicz *et al* (1986); Malendowicz (1987) in the rat; Baker (1937) in the dog and Smollich (1962) in the swamp beaver. Adrenal size differences have generally been shown to be due to differences in the adrenal cortex, particularly the zona fasciculata and not to differences in the medulla.

The process of growth and differentiation of the adrenal gland tissues goes on for several weeks after birth. Bertholet (1980) stated that the processes of growth and differentiation of the adrenal cortex and cell renewal are not yet well understood. There are two main hypotheses to explain these processes. The first, centripetal migration of cells, was proposed by Gottschaü in 1883 and is still favoured by many workers, for example; Waring (1935), Brenner (1963), Wright *et al* (1973), Wright & Voncina (1976) and Bertholet (1980). This hypothesis is based on the occurrence of cell division in the outer cortex and cell death in the inner part of the cortex. Gottschaü's suggestion is that cells migrate inward toward the inner parts of the cortex

and substitute for dead cells, but also maintain a degree of proliferative activity as they migrate. The second hypothesis was proposed by Chester Jones in 1948 and is based on the finding of DNA synthesis in all cortical zones. Chester Jones suggests that cell divisions occur in all parts of the cortex and is reluctant to accept the existence of any inwards migration.

The mammalian adrenal gland is innervated by the sympathetic nervous system, which originates at the spinal cord at the thoraco-lumbar level. Kesse *et al* (1988) stated that the adrenal gland receives both pre- and post-ganglionic sympathetic fibres. It is the pre-ganglionic fibres which innervate the chromaffin tissue of the adrenal, whereas the post-ganglionic fibres innervate structures within the cortex.

The mammalian adrenal gland is rich in blood supply. Blood vessels are arranged in a definite pattern throughout the gland.

3.1.2 Mammalian Adrenal Gland Development

Morphogenesis and differentiation of the adrenal gland start as soon as the medullary tissues migrate into the cortical anlage early in embryonic life. Gland development continues for a period of time after birth - several weeks in laboratory animals such as rats and mice, and a few years in humans. This involves cell movement, cell arrangement in the final position, cell division, maturation and differentiation of the adrenal gland tissues (cortical and chromaffin cells). As a result of these processes, the cortex is formed peripherally and the medulla internally.

The development of the mammalian adrenal gland occurs rapidly in early embryonic life and seems to slow down near the end of term (Lever, 1955). The adrenal can be considered as a single organ as soon as chromaffin cells start to migrate into

the cortical anlage. The adrenal is separated from the general mesenchyme of the kidney area as soon as its capsule is formed.

The centralisation of the chromaffin cells begins during embryonic life and goes on for several days after birth (Waring, 1935 in the mouse; Lever, 1955; Bertholet, 1980 and Verhofstad *et al*, 1985 in the rat). A large number of chromaffin cells are present in the centre of the gland by the end of embryonic life in these animals, and by the end of the first ten days after birth all chromaffin cells are concentrated in the centre of the gland. At the same time, as the chromaffin cells are becoming centralised, cortical cells are arranging themselves into zones. This occurs partly during embryonic life and goes on for several days after birth in rats (Lever, 1955; Nussdorfer, 1970 and Magalhâes *et al* 1981) and for several weeks after birth in mice (Waring, 1935) until the gland achieves the adult pattern.

The origin and the initial appearance of the adrenal's tissues have been discussed in chapter one (sections 1.3.1; 1.3.1.1; 1.3.2 and 1.3.2.1).

Since my work here is on the development of the adrenal gland in embryonic and postnatal mice, it is relevant here to review in detail some of the previous work on adrenal gland development in rodents and similar mammals. The most detailed studies I have found are by Waring, (1935) on mice; Howard-Miller, (1927) on X-zone of mice; Lever, (1955); El-Maghraby & Lever, (1980); Magalhâes *et al*, (1981) on the rats and Coupland & Weakley, (1968) on rabbits.

Most of these studies are at the light microscopic level. No previous study has looked at mouse adrenal development as a whole at the electron microscopic level.

For convenience, I will discuss previous findings on cortical tissues, chromaffin tissues, X-zone, inner capsule and outer capsule separately.

3.1.2.1 Segregation of Cortical and Chromaffin Tissues

Cortical and chromaffin tissues are intermingled during early development of the adrenal gland. Waring (1935) showed that cortical and chromaffin cells are intermingled during the first few days after the migration of chromaffin tissues into the adrenal gland of the mouse embryo. This intermingling happens on about the 13th day of gestation and persists for few days up to and including the 15th day of gestation.

The arrangement of the adrenal gland tissues (cortex and medulla) in their final positions may correspond with the differential adhesion hypothesis (DAH). The DAH was proposed by Steinberg in 1964. The DAH proposes that in a mixed population of two kinds of cells, the more cohesive cells tend to aggregate internally and therefore form an inner layer, whereas the less cohesive cells tend to aggregate externally and form an outer layer. Studies on the DAH have mainly involved unnatural mixtures of cells, but the adrenal gland is a naturally occurring case where two cell populations appear to sort out from one another in the manner suggested by the DAH. A major purpose of this study of mouse adrenal gland development was to determine to what extent adrenal cell segregation follows the prediction of Steinberg's hypothesis. This is investigated by following in detail the changes in cell distribution and shape as the adult pattern develops, and also by experimentally manipulating the gland structure. If the adrenal gland cell segregation occurs by means of DAH, then the chromaffin tissue is more cohesive, hence it

aggregates internally, whereas the cortical tissue is less cohesive, hence aggregates externally.

3.1.2.2 Cortical Tissues

In the mouse embryo, Waring (1935) studied the development of the adrenal gland, and showed that the cortical anlage is distinguishable from the surrounding mesenchyme by the closer aggregation of the cells nuclei and by the densely crowded cytoplasmic granules by about the 11-12th day of gestation. He also showed that cortical cells show a large number of mitotic figures, more than the surrounding mesenchyme.

The cortical cells are mostly present at the periphery of the adrenal throughout embryonic life but with some still present at the centre by the end of embryonic term and even after birth. Waring (1935) stated that in mouse, cortical cells begin to show some features of the future adult zonation by about the 16th day of gestation, especially the zona fasciculata. The adult zonal arrangement of the adrenal cortex is more obvious after birth. Waring (1935) and Chester Jones (1950) showed that the cortex of the mouse adrenal gland possesses three zones: zona glomerulosa; zona fasciculata and the X-zone by the 21-25th day after birth. On the other hand, Müntener & Theiler (1974) argued that the mouse cortex possesses four zones: zona glomerulosa, zona fasciculata, "zone of small cells" and the X-zone on about the 14-21st day after birth. The zona reticularis of the adult cortex appears when the X-zone starts to disappear (Waring, 1935) or after it has already disappeared according to Howard-Miller (1927). The zona reticularis is differentiated from the inner cells of the zona fasciculata according to Howard-Miller (1927)

and Waring (1935), but Müntener & Theiler (1974) claimed that it is differentiated from the "zone of small cells".

Lever (1955), Nussdorfer (1970) and Magalhâes *et al* (1978, 1981) reported that in the newly born rat the cortex possesses three zones -zona glomerulosa, zona fasciculata and zona juxtamedullaris (a group of irregular cortical cells cords which contains some medullary cells, located between the zona fasciculata and the medulla). The adult cortical zones (zona glomerulosa, fasciculata and reticularis) are well established and become more evident on the 12th day after birth according to Bertholet (1980) but Wright *et al* (1973) and Magalhâes *et al* (1981) reported that it is about the 14th day after birth that the three zones of the adult cortex are well established. On the other hand Lever (1955) claimed it is on the 21st day that the zona reticularis starts to develop and therefore the adult zones of the cortex are attained. There has not been any report of the presence of an X-zone in the developing rat adrenal.

Black (1972) indicated that the guinea-pig adrenal anlage becomes obvious on the 21-22 days (about 1/3 of the way through the 65-70 day gestation period). The adrenal gland becomes encapsulated by 27 day and zonation of the adrenal cortex starts to take place about midterm.

Luckett & Scott (1968) reported that the adrenal cortex of the rhesus monkey embryo has 2 zones; a thicker inner "fetal zone" and a narrow outer zone. The fetal zone will regress after birth and the outer zone become wider and form the definitive cortex (Luckett & Scott, 1968).

In the human embryo also, the adrenal gland has two developing cortices; the fetal and permanent cortex. The fetal cortex develops first followed by the permanent cortex which

surrounds the fetal cortex. Just prior to and after birth, the whole fetal cortex undergoes degeneration except for its outer region which contributes to the development of the future zona reticularis and at the same time the permanent cortex grows and eventually differentiates into the adult zones (Kime *et al*, 1980 and Langman, 1981). The adult cortical zones become more obvious by the time of puberty (Langman, 1981).

3.1.2.3 Chromaffin Tissues

Waring (1935) showed that in the mouse most chromaffin cells are present in the centre of the gland by the 16th day of gestation, and by the day of birth most chromaffin cells have become differentiated. The mature chromaffin cells have a high cytoplasmic to nuclear ratio. Waring (1935) reported that by the 14th day after birth, the chromaffin cells are arranged in groups separated by thin connective tissue layers and blood vessels and that this arrangement persists throughout the animal's life.

There are two types of chromaffin granules, adrenaline (A) and noradrenaline (NA). Chromaffin cells contain both kinds of granules during embryonic life but begin to differentiate into separate adrenaline (A) and noradrenaline (NA) cell types either by the end of embryonic life or in the postnatal period depending on the species.

In mouse, Jurecka *et al* (1978) found that by the end of the first week of postnatal life most chromaffin cells are differentiated into (A) and (NA) cell types and by the 3-4th week of life all chromaffin cells are fully differentiated. Coupland (1984) however, found some undifferentiated chromaffin cells in 3-6th week old mice (that is both A and NA granules were present

in the same chromaffin cell).

In most other mammals studied chromaffin cells become differentiated into (A) & (NA) cells after birth. Miller & Unsicker (1981) found that on about the 21st day of gestation of the rat embryo, chromaffin cells start to differentiate into (A) and (NA) cell types. On the other hand, Daikoku *et al* (1977), El-Maghraby & Lever (1980) and Verhofsted *et al* (1985) reported that chromaffin cells are not differentiated before birth, and that both (A) and (NA) granules are present in the same cells. According to Verhofsted *et al* (1985) the (A) and (NA) cell types can be identified on about the 2-3rd day after birth. However, El-Maghraby & Lever (1980) found that the (A) and (NA) cell types cannot be identified until the end of the first week after birth, and Daikoku *et al* (1977) reported that (A) and (NA) cells are recognisable by about the 10th day after birth.

In the rabbit, Coupland & Weakley (1968) reported that the (A) and (NA) cell types of the chromaffin cells are identifiable on about the 28th day of embryonic life, that is 1-2 days before birth.

In the guinea pig, Black (1972) showed that chromaffin cells start to migrate into the developing adrenal on the 24 day of gestation (the gestation period in the guinea pig is 65-70 days). This study did not give detail about the onset of chromaffin cell differentiation.

3.1.2.4 X-zone and Inner Capsule

The X-zone is the innermost region of the developing mouse cortex between the medulla and the zona fasciculata. It is a transitory zone and found in both immature males and virgin females. Howard-Miller (1927) studied this region in the mouse

cortex and called it the X-zone. The time of appearance, duration and disappearance of the X-zone relate to both the sex and the age of the individual (Howard-Miller, 1927). The X-zone develops several days after birth and eventually disappears by the time of puberty in the male and on the first pregnancy in female. Waring (1935) claimed that the X-zone differentiates from the original cortical anlage. On the other hand, Hirokawa & Ishikawa (1974) argued that it is not the remnant of the fetal cortex, but rather a differentiation of postnatal cells, especially those of the functional inner region of the cortex.

The X-zone cells possess dense cytoplasm. They are rich in smooth endoplasmic reticulum with a whorled form, plenty of free ribosomes, contain few lipid droplets and have mitochondria of a bizarre shape (Ross, 1967; Sato, 1968 and Hirokawa & Ishikawa, 1974, 1975).

The signs of X-zone degeneration are characterised by the collapse of the cells with gradual shrinking of the cytoplasm, collapse of the nuclei and decrease in size and number of lipid droplets (Waring, 1935; Holmes & Dickson, 1971 and Deacon *et al*, 1986).

As a result of X-zone disappearance, a connective tissue capsule is formed separating the cortex from the medulla (Waring, 1935; Holmes, 1955; Shelton & Jones, 1971; Holmes & Dickson, 1971 and Deacon *et al*, 1986).

Castration of adult male mice whose X-zone had already disappeared was found to induce the development of a similar-looking zone, termed the "secondary X-zone" (Ross, 1967; Chester Jones, 1949 and Hirokawa & Ishikawa, 1975). The secondary X-zone is considered to be differentiated from the zona reticularis

(Ross, 1967 and Hirokawa & Ishikawa, 1975).

Chester Jones (1950), Holmes (1955) and Holmes & Dickson (1971) found that hormones such as testosterone and progesterone promote the degeneration of the X-zone.

A similar zone is also found in some other species such as the rabbit, cat, red squirrel, voles and golden hamster but not in others, such as the rat and humans (for review see Deacon *et al*, 1986).

3.1.2.5 Outer Capsule

In the mouse, the adrenal glands are clearly separated from the general mesenchyme on about the 13th day of gestation by a well marked mesodermal sheath (Waring, 1935). Waring (1935) claimed that by this time the gland can be dissected out as a discrete entity. On about the 14th day of gestation the glands have a well marked capsule of connective tissue cells and fibres. Capsule development seems not to have been studied systematically in other mammals.

3.1.2.6 The Aims of This Study

The main aims of the study of mouse adrenal development are as follows:

1. To examine the morphogenetic events that take place during adrenal development using light and electron microscopy. By means of examining the structure of the cells to gather clues concerning the means by which morphogenetic processes are occurring in the adrenal gland. In particular, the shape of the cells, and their junctions with their neighbours, might suggest active movement or differential adhesiveness as the adrenal tissues sort out from one

another.

2. To clear up minor discrepancies in previous accounts, such timing of medullary cell differentiation, and cortical cell zonation.
3. To examine in detail the development of the inner and outer capsules.
4. To examine in detail the appearance and disappearance of the X-zone. Also to look for any other relevant features about the X-zone.

3.2 Results

3.2.1 Light Microscopy (LM)

3.2.1.1 Embryonic Tissues (13-18th day)

The mouse adrenal glands vary in their shapes and sizes even in the same embryo. Some adrenals have a triangular shape, others are oval-round and some are somewhat elongated, though the oval-round shape is frequent.

The mouse adrenal gland is a loose structure during its early development. The two main adrenal tissues are intermingled with each other and show little sign of the eventual adult adrenal organisation. It is near the end of embryonic term that the two main tissues of the adrenal take their final positions. That is when the chromaffin tissue is concentrated at the central region of the gland forming the medulla, and the cortical tissue confined to the peripheral region forming the cortex.

The following description of mouse embryonic adrenal glands is based on 1 µm semithin araldite sections prepared as described in section 2.2, 2.2.1, 2.2.2, 2.2.2.1; except for adrenals of 13

day mouse embryos, which of 6 ʌm thick wax sections. Sections of all stages, except 13 day, were stained routinely with toluidine blue, where chromaffin cells are stained light blue and cortical cells are dark blue. When sections were stained with ammoniacal silver carbonate, chromaffin cells are stained light brown and the cortical cells are dark brown. The 13 day specimens were stained with H&E, where chromaffin cells nuclei are stained darker and cortical cells nuclei are lighter. For convenience, the two main cell types are considered separately, followed by accounts of cell death, the outer capsule, cell division and other elements of the adrenal.

3.2.1.1.1 Cortical Tissue

The 13 day mouse embryo adrenal is a loose structure (fig 3.1). Cortical cells have light stained nuclei and dark cytoplasm. The cortical tissue makes up the major part of the adrenal gland (fig 3.1). Cells have a polygonal shape with polygonal nuclei.

The cortical cells at 14-15 days are intermingled with the chromaffin cells of the adrenal and do not show the organisation characteristic of the adult cortex (fig 3.2). Most cortical cells have a polygonal shape with round to oval nuclei (up to 4 nucleoli). A very few cortical cells have elongated nuclei, the nuclei having an undulating outer shape. At the light microscopic level, no cortical cells appear to have elongated locomotory processes.

By the 16-18th day, the cortical cells have taken the peripheral position. Cells have a similar appearance to those of the 14th-15th day. Some cortical cells start to show some sort of cellular arrangement which resembles the future adult zones,

especially the zona fasciculata. This is seen on the 17th day and become obvious on the 18th day, when cells start to be arranged in a fascicular pattern (fig 3.3). Some cortical cells show some vacuoles within their cytoplasm which represent lipid droplets. Those cells are seen mainly at the periphery of the gland (fig 3.4). There are some cortical cells (single cells and a few islets) still present among the chromaffin tissue at the centre of the gland (fig 3.5). The cells of the islets have a polygonal shape, but some of the single cells have an elongated shape (fig 3.5). At the same time some chromaffin islets are present within the cortical tissue outside the central region (fig 3.3).

Quantitative measurements of the volume of the cortical and chromaffin tissues and the relative position of the medulla will be given in chapter 7.

3.2.1.1.2 Chromaffin Tissue

In the 13 day mouse embryo, a group of cells associated with nerve fibres were seen located outside the gland's capsule (fig 3.1). These cells have darker nuclei with light cytoplasm resembling the chromaffin cells of later stages and adult. Cells of this type are almost entirely occupied by large nuclei with very little cytoplasm (fig 3.1). They have a polygonal shape and are seen on both sides of the developing capsule, and all around the gland. They are not seen deep within the gland. I assume that these are presumptive chromaffin cells migrating into the gland. A few appear to have an elongated shape and look as if they have locomotory processes (fig 3.1). Most cells look as if they are grouped in clumps rather than being isolated. These cells are few in number when compared with those of the cortical tissue type.

By the 14th day, chromaffin cells were seen in isolated groups which are intermingled with the cortical tissue throughout the adrenal (fig 3.6). These chromaffin groups vary in size; some are large (about 18 cells); others are small (up to 2-4 cells). A few single chromaffin cells were seen among the peripheral cortical cells near the capsule.

The intention of this work is to study the morphology of the adrenal tissues within the boundary of the gland's capsule. By the 14th day of gestation the gland is encapsulated and therefore it was dissected out without the surrounding tissues. It may be that some migrating prospective chromaffin cells do not enter the gland before encapsulation, and are therefore excluded, but I have no clear evidence for this.

There are two types of chromaffin cell present; some have a high nuclear to cytoplasmic ratio whereas the others are the opposite. The majority of the chromaffin cells are polygonal and have polygonal nuclei. Some chromaffin cells have elongated nuclei. The orientation of the elongation varies from cell to cell. These cells with elongated nuclei are present mainly within clumps. It is hard to tell with the LM if the complete cells are really elongated or not.

In the 15-16 day mouse embryo, a large number of chromaffin cells have reached the centre of the gland (fig 3.2). Some groups of chromaffin cells were still scattered among the cortical tissue out of the central region of the gland with about 2-8 cells within these groups (fig 3.2). Those chromaffin cells which have reached the centre are arranged in large groups (more than 30 cells). They are seen connected to each other except for some which are separated by cortical cells. Most chromaffin cells have a polygonal shape with round to oval nuclei. Cells with

elongated nuclei are present in both large and small groups. Those within the large groups are seen mainly at the periphery of the group near the cortical tissue side. The orientation of the nuclei of these cells suggests they have recently joined the group they are now part of. They are orientated as if they are moving towards the centre of the group.

By the 17-18th day, most chromaffin cells have reached the centre of the gland with the exception of small islets of chromaffin cells which are seen among the cortical cells around the central region (about 2-6 cells in each islet), but not far from the centre (fig 3.3). The chromaffin cells have a polygonal shape with round to oval nuclei. There are still a few cells with elongated nuclei cells present in the chromaffin tissue. They are mainly seen at the periphery of the medulla and are orientated towards the centre. Occasionally cells with elongated nuclei are present near the centre of chromaffin cell groups. A few small islets of cortical cells are present in the centre of the gland (about 1-6 cells in each islet) intermingling with those of the chromaffin type. These cortical cells have both elongated and polygonal nuclei (fig 3.5), but the elongated nuclei have no particular orientation.

3.2.1.1.3 Cell Death

Cells showing signs of dying occur during embryonic life. They stain very densely, have a polygonal shape and very irregular dark nuclei. They mostly occur in small groups, but also singly. It is not possible to identify with light microscopy whether they are cortical or chromaffin cells. They look very vacuolated and have a spongy appearance (fig 3.4, 3.7). These

cells are present in all stages of mouse embryos that have been studied in this work (13-18 day mouse embryos). These dark cells are seen throughout the gland. They are not confined to a particular area and are seen in the central and in the peripheral region (fig 3.2, 3.4, 3.6, 3.7). These dying cells are seen among cortical and also chromaffin tissues. A count was made of these dying cells using serial semithin araldite sections based on light microscopic observations. These results are presented more fully in chapter 7. The pattern of cell death in postnatal mouse adrenals is very different, and is described in section 3.2.1.2.5.

To my knowledge cell death in embryonic mouse adrenal has not been reported previously.

3.2.1.1.4 Outer Capsule

The adrenal gland's capsule of the 13 day embryo is not well established. Cells that make up the capsule have a polygonal shape and also have a high nuclear to cytoplasmic ratio (fig. 3.1). There are very few flattened cells and also few connective tissue fibres (fig 3.1). The capsule is about 2-5 cells thick in the 14-15 day embryo. At this stage the majority of capsule cells are flattened but loosely organised. In the 16-18 day mouse embryo the capsule is about 3 to 8 cells thick. At this stages the capsule cells becoming more compactly organised and connective tissues fibres are present. Measurements of the capsule thickness of both embryonic and postnatal glands are presented in Table 3.1.

3.2.1.1.5 Mitosis

Mitotic figures are frequently seen in both the cortical and chromaffin tissues though they are more frequent within the cortical than chromaffin. Mitotic counts were made on three stages: 15 and 17 day mouse embryos and 1 week postnatal mouse, using serial semithin araldite sections. Mitosis is more frequent in cortical than chromaffin tissue. In cortical tissue, mitotic figures were seen mostly at the periphery with fewer more centrally. Mitotic index data is presented in detail in chapter 7.

3.2.1.1.6 Other Elements within the Adrenal

There are other elements present in the adrenal gland which include blood vessels and nerves.

The adrenal gland is rich in blood supply. Blood cells are present in the 13th day mouse embryo adrenal gland (6 um thick sections). They are seen scattered throughout the gland tissues presumably in very thin - walled vessels and also within a few more major blood vessels. More blood cells are seen from the 14th day onward. Major blood vessels become more obvious from the 15th day onward.

Nerve fibres are seen within the adrenal gland by the 14th day of gestation (fig 3.6). The nerve fibres seen are in association with the chromaffin tissue. I have not seen nerve fibres in association with cortical tissue.

3.2.1.2 Postnatal Tissues (1-35 day)

The mouse adrenal gland enlarges considerably during this period. The main tissues of the adrenal gland become more organised in their final position and take up the adult characteristics, hence the terms cortex and medulla can be used.

The adrenal glands have variable shapes and sizes even in the same animal, as is the case in embryonic stages.

The following description is based on 1 um semithin sections of mouse postnatal adrenal glands (1-10 and 14, 21, 32 and 35 days).

3.2.1.2.1 Cortical Tissue (Cortex)

The cortical tissue of the newly born mouse shows some of the adult arrangement, especially the zona glomerulosa and the zona fasciculata. It will take several weeks until the cortex is fully adult. Cortical cells are polygonal with round to ovoid nuclei. There are no more signs of cells with elongated nuclei.

By day 1 some peripheral cortical cells which are located adjacent to the capsule are arranged in cords, resembling the adult zona glomerulosa (fig 3.8). There are also some peripheral cortical cells which are arranged in fascicles, resembling the adult zona fasciculata. It is a very small proportion of these cells that are so arranged at this stage. The two developing zones are not well established at this stage, but become more obvious at day 4 after birth (fig 3.9).

A class of small cells is present at day 1 up to day 7, scattered among the developing zona glomerulosa cells, also at the boundary between the medulla and the cortex and within the medulla itself (fig 3.9, 3.10, 3.15, 3.18). Most of these cells are present in groups but do not show any signs of cell attachment to the neighbouring cells, hence are seen as individuals. These cells have polygonal shaped nuclei and a high nuclear to cytoplasmic ratio, and some show mitotic activity. These cells are seen up until the end of the first week, but not

thereafter. These cells have not been described in any of the previous reports of adrenal development. I could not identify these cells definitely, but they could perhaps be macrophages clearing up dead cells from embryonic stages. Similar looking cells are seen within blood vessels of the gland.

By the end of the first week after birth, the zona glomerulosa and fasciculata have become well established (fig 3.11). At the same time, a group of darkly - stained cortical cells lies at the innermost part of the cortex, interdigitating with both zona fasciculata cells and the chromaffin cells of the medulla. This layer of cortical cells will be referred to as the X-zone (see later for more details) (fig 3.11). Therefore the adrenal cortex of 1 week old mice possesses three zones (zona glomerulosa, zona fasciculata and the X-zone).

During the 2nd and 3rd week of life, the cortex still has the three zones which have been described at the end of the 1st week (fig 3.12). The zona glomerulosa and fasciculata are well developed and are much bigger than those at 1 week. The cells of the developing zona reticularis start to appear under the zona fasciculata around 32 days while the X-zone is undergoing degeneration (fig 3.13).

By the 5th week, the male adrenal cortex is morphologically equivalent to that of the adult cortex. The cortex now has the three adult zones; glomerulosa, fasciculata and reticularis (fig 3.14). The zona reticularis has polygonal cells with almost round nuclei. Cells of the zona reticularis have an anastomosing pattern. By this time, the X-zone has degenerated in the male (see later section 3.2.1.2.5) and as a result the cortico-medullary capsule has been established (fig 3.14). On the other hand, the female adrenal cortex still possesses the X-zone until

much later.

3.2.1.2.2 Chromaffin Tissue (Medulla)

The medulla of the adrenal varies somewhat in its location within the gland. It does not always form exactly in the middle of the gland. Details on medulla position will be given in chapter 7. The medulla varies in overall shape from round to oval. The boundary between the cortex and medulla is undulating at the start of postnatal life.

The chromaffin cells are polygonal in shape with almost round nuclei (2-3 nucleoli). By day 1 of postnatal life, most chromaffin cells are concentrated in the medulla (fig 3.15). There are still a few small islets of chromaffin cells within the cortical tissue close to the medulla. By this time there are no more single, isolated chromaffin cells within the cortical area. The shape of these cells in small clumps is not significantly different from those already in the medulla. Cells with elongated nuclei are not seen.

By about day 4, some chromaffin cells are arranged in cords within the medulla and separated by thin connective tissue layers and blood sinusoids from each other (fig 3.10). By the end of the 1st week, the chromaffin cells of the medulla have assumed their final position in the centre of the gland (fig 3.16). More chromaffin cells are arranged in cords by the end of the 1st week of life and no chromaffin cells are left in the cortical area (fig 3.16). The chromaffin cells at 2-3 weeks are similar to those seen in the 1st week except that more chromaffin cells are now arranged in cords (fig 3.17). By the 5th week after birth the chromaffin cells of the medulla of the male adrenal gland have

achieved their adult characteristics: almost all chromaffin cells are now arranged in cords (fig 3.14).

On the basis of their staining reaction with toluidine blue, there are two types of chromaffin cell, lightly and darkly stained, with darkly stained cells being in the majority (fig 3.17). This differentiation of cells is seen in the 1 day postnatal medulla (fig 3.18). Although it is not possible on the evidence of toluidine blue stains to discriminate between chromaffin granule types, on the basis of previous reports (Lever, 1955; Coupland *et al*, 1964 and El-Maghraby & Lever, 1980) light cells contain (A) granules and dark cells contain (NA) granules, or undifferentiated containing (A) and (NA) granules. The two cell types are randomly intermingled with each other in the medulla. By the end of 1 week postnatal, the two cell types of the medulla (dark and light cells) have become more obvious (fig 3.16). The dark cells are still in the majority, but the light cells are increasing in frequency compared to the previous stages. Both cell types are intermingled with each other in the medulla. More light chromaffin cells are present by 2-3 weeks and are arranged in cords. Both light and dark cells are arranged in cords separated by a thin connective tissue layer on the 2nd week after birth (fig 3.17). The arrangement of the two cell types in the medulla is no longer entirely random. Light cells tend to be more frequent at the periphery of the medulla, and many cords contain only one cell type, dark or light. There are, however, also cords which contain both cell types.

3.2.1.2.3 Outer Capsule

The gland's outer capsule gets thicker as the animal grows. It is 3-8 cells thick by the end of the first week and become

thicker in older mice. It is 6-10 cells thick in 3 and 5 week old mice. Measurements of outer capsule thickness in both embryonic and postnatal adrenals are shown in Table 3.1.

3.2.1.2.4 Mitosis

Mitotic figures are frequently seen in both cortical and chromaffin tissues. They are more frequent within the cortical tissue than the chromaffin tissue. In the cortical tissue they are mainly seen in the periphery of the cortex especially in the zona glomerulosa and outer zona fasciculata. These data are presented in detail in chapter 7.

3.2.1.2.5 The X-Zone and the Inner Capsule

The X-zone is a group of transitory cortical cells which appears in postnatal life as a distinct layer; it disappears at the time of puberty in both males and females. It develops very early in postnatal life (about day 4) as scattered cells at the interface of the medulla and cortex (fig 3.10). Cells of the X-zone are stained darker than the rest of the cortical cells. The cells are polygonal in shape with almost round nuclei. They have few lipid droplets.

By the end of the first week after birth, cells of the X-zone start to be arranged in a thin layer between the cortex and medulla (fig 3.16). The boundary of the X-zone with the medulla and zona fasciculata is not smooth but interdigitated.

The X-zone becomes wider in the 2nd and 3rd week of life (fig 3.12). The X-zone reaches its maximum width at week 3. Measurements of changes in X-zone width are shown in Table 3.2. At least part of this growth in the X-zone is due to cell

proliferation in the outer part of the X-zone (fig 3.19) (which borders the zona fasciculata), but I saw no cell division in the inner part of the X-zone (which borders the medulla). The tissue of the X-zone can be divided into two layers with respect to its appearance (fig 3.12): a more compact outer layer, located near the zona fasciculata, and a looser inner layer bordering the medulla. These two layers become more obvious by 3 weeks. A demarcation line starts to develop between the inner and the outer layers at about the 3rd week and becomes well established in 32 day old mice, both in males and females (fig 3.13). The outer layer consists of healthy cells which have a polygonal shape and almost round nuclei. These are the developing zona reticularis cells. On the other hand, the inner layer becomes very loose, especially in the male mouse. By the 35th day after birth, the male X-zone has almost disappeared whereas that of the female of the same age is still present. The female X-zone delays degeneration until some point during the first pregnancy. I routinely fixed the adrenals of the pregnant adult females from which I took embryos. Sections of these showed the presence of the X-zone at least until 16 days of the first pregnancy (fig 3.20).

As a result of X-zone degeneration, a capsule develops between the medulla and the cortex (fig 3.14). It will be referred to as **inner caspule** (cortico-medullary capsule). The capsule is made up of dead cells of the inner X-zone layer and some connective tissue fibres. The outer X-zone layer becomes the zona reticularis (fig 3.14). The cortico-medullary boundary has a smooth, but undulating shape (fig 3.14). Signs of cell death at the light microscopic level include the loss of contact between neighbouring cells, collapse of cells and the presence of empty

spaces between cells of this zone (fig 3.13, 3.21). The degeneration of the X-zone occurs mainly on the medullary side.

3.2.2 Transmission Electron Microscopy (TEM)

It is not the objective of this study to give a detailed ultrastructural account of the adrenal gland. Such accounts are readily available else where (Coupland, 1965a). After brief accounts of the main ultrastructural features of the main cell types, I will concentrate on features of relevance to gland morphogenesis. These features include changes in cell shape, distribution of cell junctions, presence of microfilaments and microtubules, signs of cell death, and the timing of chromaffin cell differentiation.

The two main tissues of the adrenal gland differ in their ultrastructural features. The main features of the cortical cells are: the abundance of mitochondria, smooth endoplasmic reticulum (SER) and the presence of lipid droplets. On the other hand, the main features of the chromaffin cells are the numerous membrane-bound cytoplasmic secretory granules and rough endoplasmic reticulum (RER).

3.2.2.1 Cortical Tissue

Most cortical cells have a polygonal shape. In embryonic life the cortical cells have polygonal nuclei, whereas in postnatal cells they are almost round. The cortical cells have many mitochondria and abundant SER. They also have many free ribosomes which in some are arranged in polysomes. The Golgi complex is also present. All these features are seen from the 14th day of gestation onwards (fig 3.22).

Lipid droplets are very scarce in the early stages. They become obvious by the 16th day of gestation (fig 3.23), and increase in number near the end of embryonic life. They become abundant in all the cortical zones of the postnatal adrenal gland (fig 3.24). The amount of lipid droplets differ in the different zones of the cortex. Cells of the zona fasciculata have the most abundant lipid droplets, followed by the zona glomerulosa cells and then the zona reticularis.

Features associated with cell shape and motility are described in section 3.2.2.3.

3.2.2.2 Chromaffin Tissue

Most chromaffin cells have a polygonal shape with polygonal nuclei during embryonic life. The nuclei become round to oval in postnatal life. Each nucleus has about 1-3 nucleoli which are close to the nuclear membrane. There are heterochromatin patches near the nuclear membrane.

The chromaffin granules vary in their electron density. They either have a dense or a moderately dense core (fig 3.25, 3.26, 3.27). The granules are observed as early as the 14th day of gestation (fig 3.22), and are mainly of the dense-cored type. According to Coupland *et al* (1964); Coupland (1965a) and Singh & Mathew (1987) dense-cored granules contain noradrenaline (NA) and moderately dense ones adrenaline (A). In 17-18 day embryos, chromaffin cells contain both types of granules, though the dense-cored ones are more frequent. That means that chromaffin cells have not yet differentiated. Differentiation of chromaffin cells into adrenaline and noradrenaline cell types takes place during postnatal life. This is seen by day 1 onward and by day 14 the majority of chromaffin cells were differentiated into A and

NA cell types, though some chromaffin cells still possess the two granules. The chromaffin granules are scattered throughout the cytoplasm with a tendency to a peripheral location. The granules increase in number in each cell as the embryo gets near the end of embryonic life and also in postnatal life. A few dense and moderately cored granules show attachment to the plasma membrane of the cell during embryonic life (fig 3.24).

The cytoplasm of the chromaffin cells also possesses RER, mitochondria, Golgi complex, free ribosomes, microtubules and microfilaments. Features associated with cell motility are described in section 3.2.2.3. All of these ultrastructural details can be seen on the 14th day of gestation onward (fig 3.22, 3.26, 3.28).

The RER has variable shapes and sizes. Some RER are very dilated and have a bizarre appearance and some are seen associated with the nuclear membrane (fig 3.28). This phenomenon is also seen in both embryonic and postnatal cells.

3.2.2.3 Small Cells

The TEM work on small cells, which had been described in light microscopy, shows that this type of cells are some type of blood cells, probably of white blood cells type (fig 3.29).

3.2.2.4 Morphogenetic Features

This section gives details about microtubules (MT), microfilaments (MF), cell surface shape and junctions. For convenience these are divided into sub-sections.

3.2.2.4.1 Cell Junctions

The cell junctions are well preserved in both cortical and chromaffin cells. Tight junctions, desmosomes and gap junctions are all present between like cells and unlike cells.

In embryonic life, tight junctions, desmosomes and gap junctions as well as intercellular spaces are present between like cells and unlike cells (fig 3.22, 3.25, 3.30). In some sections the intercellular spaces look moderately wide, whereas in others they are narrow. The intercellular spaces measure between 1.2-60 nanometer (nm) between both chromaffin and cortical cells.

In postnatal life, desmosomes as well as gap junctions are present linking both cortical and chromaffin cells of the medulla. Intercellular spaces are present but not as extensive as in embryonic life. They are seen more between zona reticularis cells and measure between 6.4 nm and 64 nm.

3.2.2.4.2 Microtubules (MT) and Microfilaments (MF)

Microtubules and microfilaments are present within the cytoplasm of chromaffin and cortical cells of all embryonic stages studied and also in postnatal stages. They are seen at the periphery of the cells and also around the nuclei. Both MT and MF may be associated with the cells membranes (fig 3.31).

At the embryonic stages that I have examined, MT and MF appear to be more abundant in chromaffin cells than cortical cells. This appearance may, however, be partly artefactual because cortical cells have very dense, darkly staining cytoplasm, which makes MT and MF difficult to distinguish. As far as I can tell, the abundance of MT and MF in chromaffin cells remains more or less constant throughout the embryonic period.

In cells with elongated nuclei, MTs seem to be orientated parallel to the long axis of the nucleus (fig 3.31). MTs with an opposite orientation are not seen. MFs are also seen orientated parallel to the long axis of the nucleus, but it is not possible to tell in conventional sections that MF do not have other orientation too.

In postnatal adrenals, chromaffin and cortical cell MT and MF were rarely seen, but presumably not absent. Fig (3.27) shows MT seen in a chromaffin cell at 2 weeks old.

3.2.2.4.3 Cell Surface Shape

The cell surface is predominantly straight and smooth, both between like cells (chromaffin-chromaffin or cortical-cortical) and unlike cells (chromaffin-cortical) at all embryonic and postnatal stages studied (fig 3.22).

There are occasional areas of convoluted interdigitating cell surface, which are seen more at early stages in both cortical and chromaffin cells, but I have not seen signs of the sort of elongated cell processes that might be expected in actively moving cells. This applies also to cells with elongated nuclei. They have an elongated overall shape, but appear to lack long thin processes.

3.2.2.5 Cell Death

The TEM work confirms the presence of dying cells in both embryonic and postnatal life.

In embryonic life, dying cells are present during the 14-18 day period throughout the gland (fig 3.32, 3.33). They are characterised by darkly stained cells with very darkly stained

nuclei. They also have an irregular shape with some nuclei being very shrunken and folded (fig 3.32, 3.33). The cells have vacuolated cytoplasm and their cytoplasmic organelles, especially the mitochondria and SER have a bizarre appearance. It is clear from examination of the cytoplasm of the dying cells that cell death occurs amongst both cortical and chromaffin cells. Occasionally dying chromaffin cells were found among cortical cells and vice versa, but most of the dying cells were seen among cells of their own type.

In postnatal life, dead cells are seen only in the inner layer of the X-zone (fig 3.34, 3.35). Signs of degeneration in the X-zone are seen as early as the 1 week and become more obvious on the 2nd and the 3rd week after birth. These degenerating cells of the postnatal cortex have similar features to those of embryonic cells.

3.2.2.6 The Inner Capsule

The TEM reveals the ultrastructure of the cortico-medullary capsule. It shows that it is made up of connective tissue fibres including collagen and debris of the degenerating X-zone cells (fig 3.36).

3.2.3 Scanning Electron Microscopy (SEM)

The aim of the SEM work was to examine cell shape and surface structures of the chromaffin and cortical cells during the period when they were moving, and to help determine which cells were moving actively and if they had the appearance characteristic of other moving cells.

This work was attempted on both embryonic and some young adult adrenal glands.

Several methods of fixing and breaking open the gland, so as to expose internal cells, were tried. Glands were broken prior to fixation or after fixation just before osmium treatment, or after osmium treatment, or finally after critical point drying.

Unfortunately, the cells of interest did not show up with any of the fracture methods used. This is perhaps because of the compactness of the internal structure of the adrenal gland tissues.

It seems unlikely that failure was due to poor fixation or fracturing technique as some tissues of the adrenal gave a clear appearance, particularly the blood vessels and cells (fig 3.37). The cortical and chromaffin tissues showed up a solid areas, with poorly demarcated cell boundaries. This was true even of early embryonic tissue (14 and 15 day) where I expected cell boundaries to be distinct. It was possible to distinguish the dividing line between cortex and medulla in the 18 day embryonic and young adult adrenals, but this gave no new information. It was also possible to distinguish the outer capsule and the inner capsule.

On the other hand, the blood vessels and cells are well preserved (fig 3.37). The endothelial cells of the blood vessels are flat and appeared to be overlapping (fig 3.37). The cell boundaries easily noted and the cellular attachment between endothelial cells are provided with microvilli which reach over neighbouring cells.

The outer capsule is seen as very compact and made up of flattened cells and connective tissue fibres (fig 3.38).

The inner capsule (cortico-medullary capsule) of the young adult adrenal is also quite clear, as a separating layer (fig

3.39), made up of connective tissue fibres.

Fig. 3.1a. Transverse section through the trunk of 13 dme showing the developing adrenal gland (AG) above the developing kidney (K). Presumptive chromaffin cells are shown in the (box). The capsule is shown as a loose structure (arrows). (Bar=100 μm).

Fig. 3.1b. An enlargement of the box in fig 3.1a, showing a group of the presumptive chromaffin cells (arrows). Chromaffin cells are known to have dark stained nuclei with light stained cytoplasm. (Bar=25 μm).

Fig. 3.2. Transverse section through 15 dme adrenal gland showing cortical (C) and chromaffin cells (m). Dying cells are indicated by arrows. (Bar=100 μm).

Fig. 3.3. Transverse section through 18 dme showing cortical (C) and chromaffin cells (m). Some cortical cells are arranged in the same sort of way as the future zona fasciculata (+). (Bar=50 μm).

Fig. 3.1. wax sections, stained with H&E.

Fig. 3.2 & 3.3. araldite semithin sections stained with toluidine blue.

AG=adrenal gland, c=cortical cells, dme=day mouse embryo, m=chromaffin cells, K=kidney, L=liver, X=gland's capsule

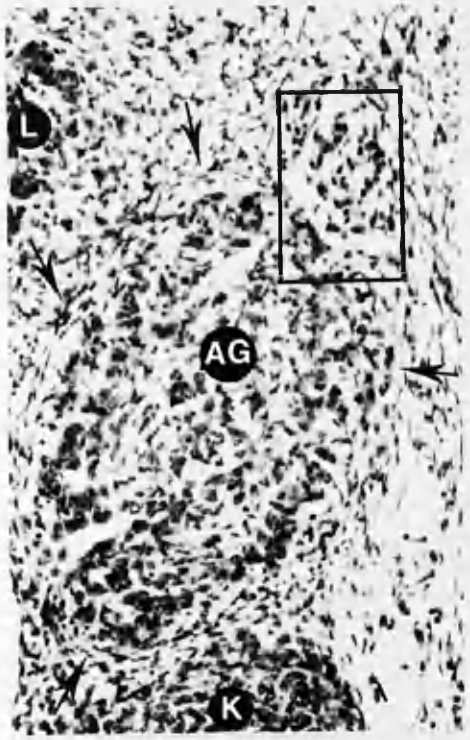


Fig.3.1a.

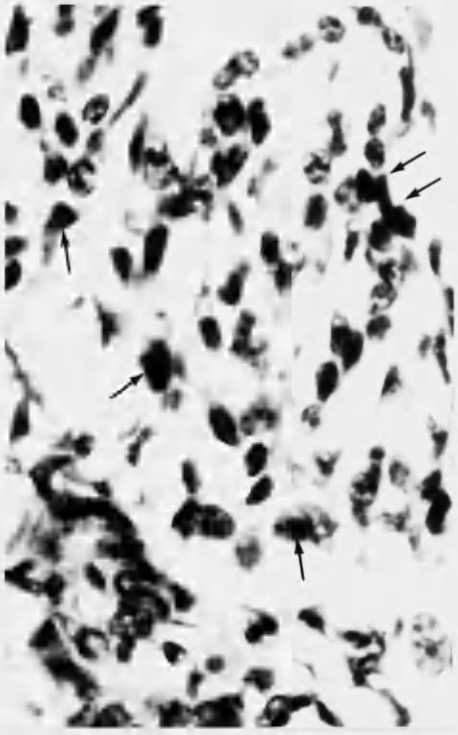


Fig.3.1b.

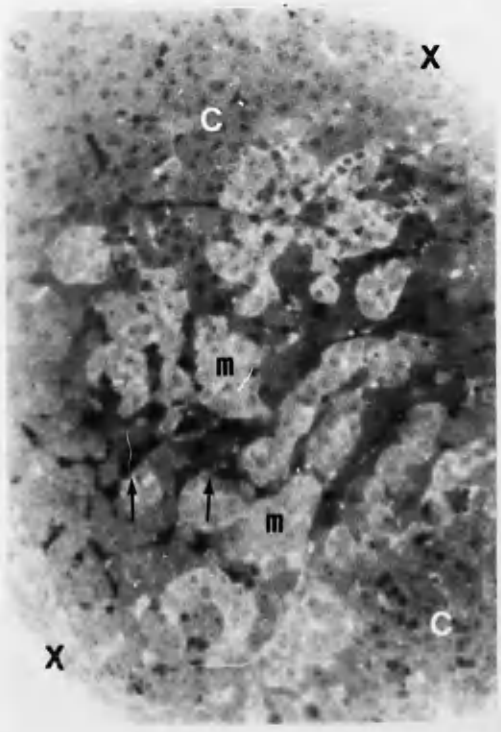


Fig.3.2.

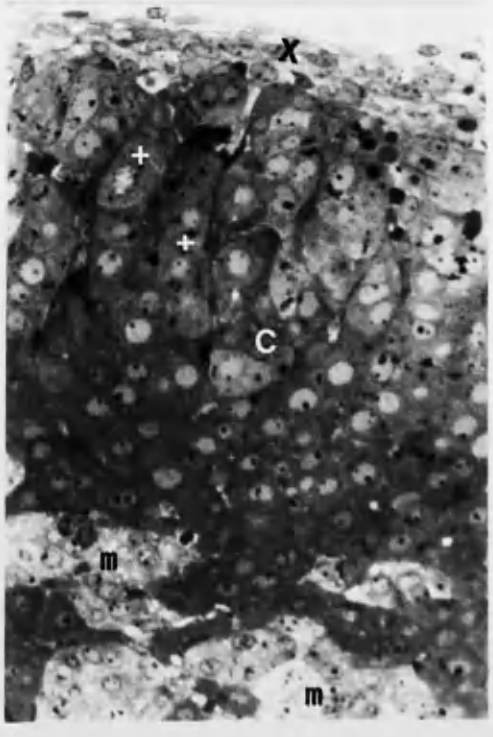


Fig.3.3.

Fig. 3.4. Transverse section through cortical tissue of 18 dme adrenal showing the presence of lipid droplets within the cortical cells (small arrows). Dying cells are indicated by large arrows. (Bar=25 μm).

Fig. 3.5. Transverse section through 17 dme adrenal gland showing most chromaffin cells at the centre (m). Islets and single cortical cells are still present among the chromaffin cells (arrows). (Bar=50 μm).

Fig. 3.6. Transverse section through 14 dme adrenal gland showing chromaffin (m) and cortical cells (c). This shows nerve fibres (large arrow) associated with the chromaffin tissue. Dying cells are indicated by small arrows. (Bar=100 μm).

Fig. 3.7. Transverse section through 15 dme adrenal gland showing cortical (c), chromaffin (m) and dying cells (arrows). (Bar=50 μm).

All of the above figures are of araldite semithin sections stained with toluidine blue.

c=cortical cells, dme=day mouse embryo, m=chromaffin cells, X=gland's capsule

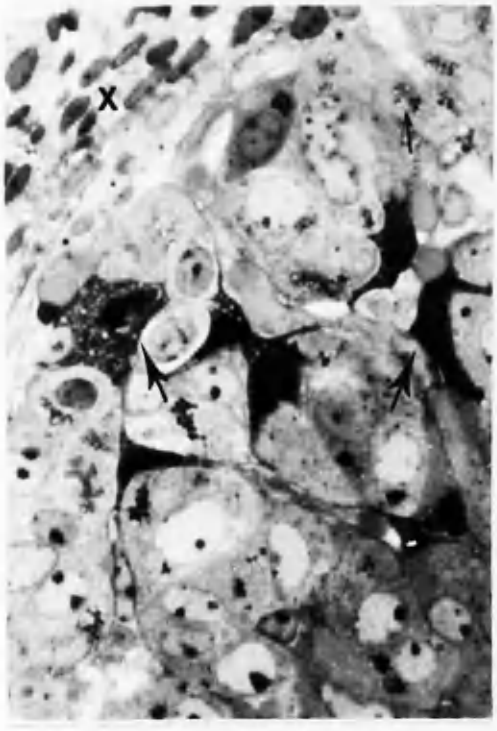
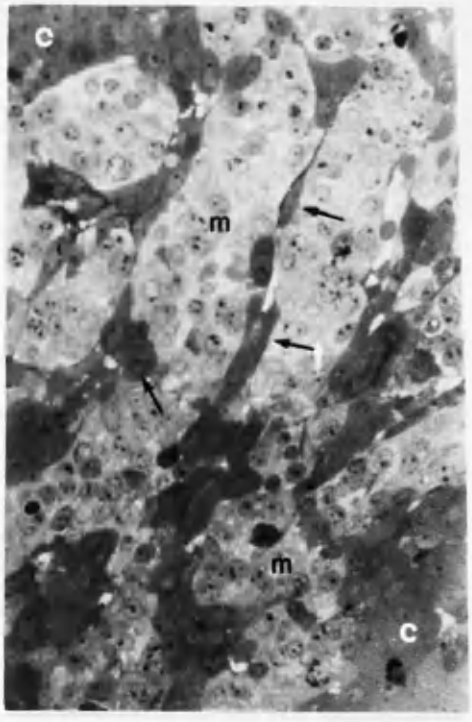


Fig.3.4.



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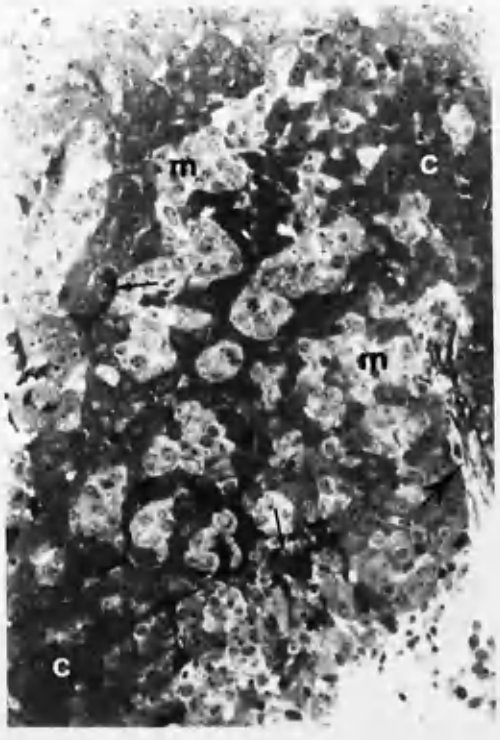

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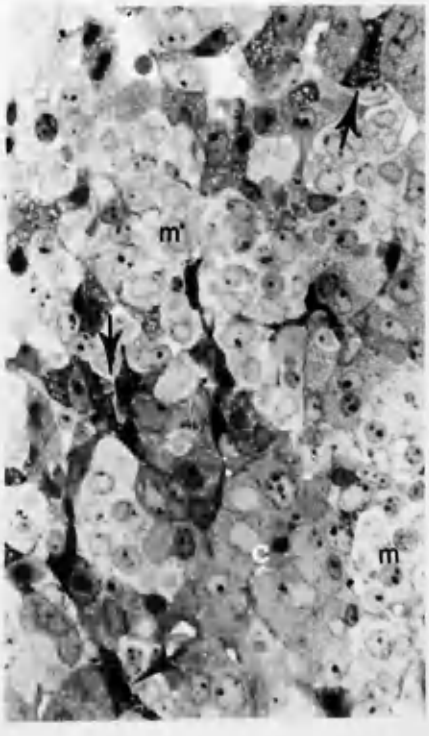


Fig.3.7

Fig. 3.8. Transverse section through 1 dpm adrenal gland showing the developing zona glomerulosa (ZG). (Bar=50 µm).

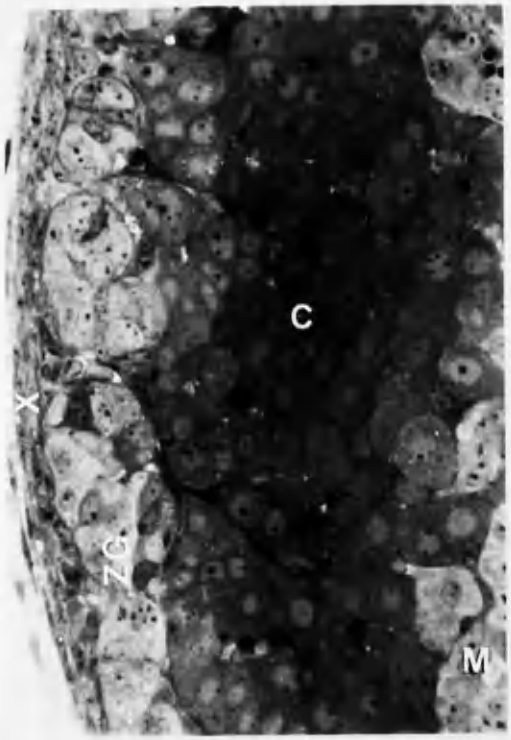
Fig. 3.9. Transverse section through 4 dpm adrenal gland showing the developing zona glomerulosa (ZG) and zona fasciculata (ZF). The "small cells" are indicated by arrows heads. (Bar=25 µm).

Fig. 3.10. Transverse section through 4 dpm adrenal gland showing groups of "small cells" (white arrows heads) in both cortical and chromaffin tissues (M). Few chromaffin cells are arranged in cords. Developing X-zone (as a thin layer of cells) is indicated by a large arrow. (Bar=50 µm).

Fig. 3.11. Transverse section through 1 wpm adrenal gland showing zona glomerulosa (ZG), zona fasciculata (ZF) and the developing X-zone as thin layer of cells (arrow). (Bar=50 µm).

All of the above figures are of araldite semithin sections stained with toluidine blue.

dpm=day postnatal mouse, M=medulla, wpm=week postnatal mouse, X=gland's capsule, ZF=zona fasciculata, ZG=zona glomerulosa



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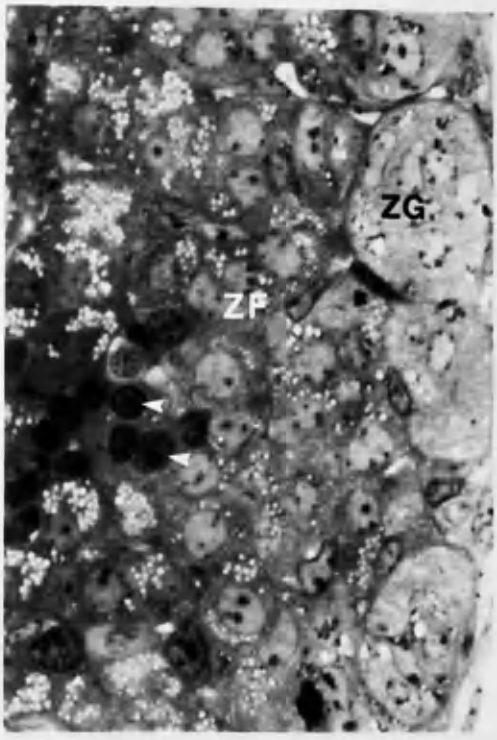

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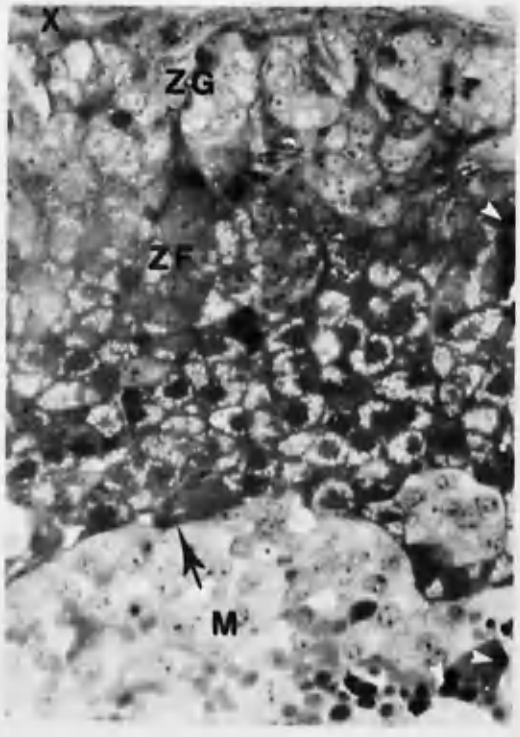

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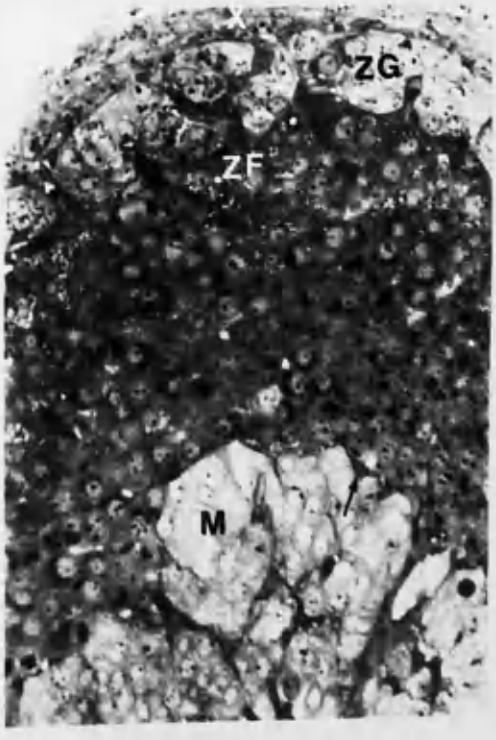


Fig.3.11.

Fig. 3.12. Transverse section through 2 wpm adrenal gland showing zona glomerulosa(ZG), zona fasciculata (ZF) and a thick X-zone (XZ). (Bar=100 μm).

Fig. 3.13. Transverse section through 32 dpm male showing the developing zona reticularis (ZR), the demarcation line (arrows heads) between the degenerating X-zone (XZ) and the developing zona reticularis (ZR). It also shows signs of degenerating X-zone (XZ). (Bar=50 μm).

Fig. 3.14. Transverse section through 5 wpm male adrenal gland showing adult organisation: the X-zone has already disappeared, and been replaced by the inner capsule (arrows) separating medulla (M) from the cortex (C). (Bar=100 μm).

Fig. 3.15. Transverse section through 1 dpm adrenal gland showing almost all chromaffin cells (M) at the centre of the gland. A group of small cells (small arrows) and cortical cells (large arrows) are present among chromaffin cells. (Bar=100 μm).

All of the above figures are of araldite semithin sections stained with toluidine blue.

C=cortical tissues, dpm= day postnatal mouse, M= medulla, wpm= week postnatal mouse, XZ=X-zone, ZF=zona fasciculata, ZG=zona glomerulosa, ZR=zona reticularis

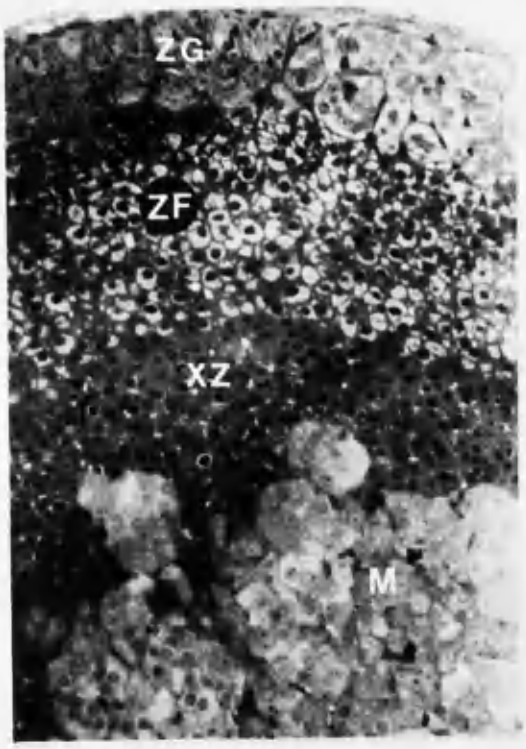
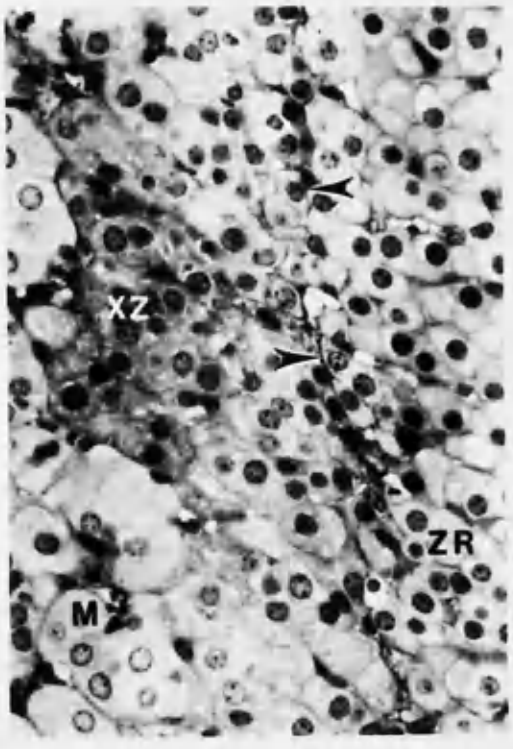


Fig. 3.12.



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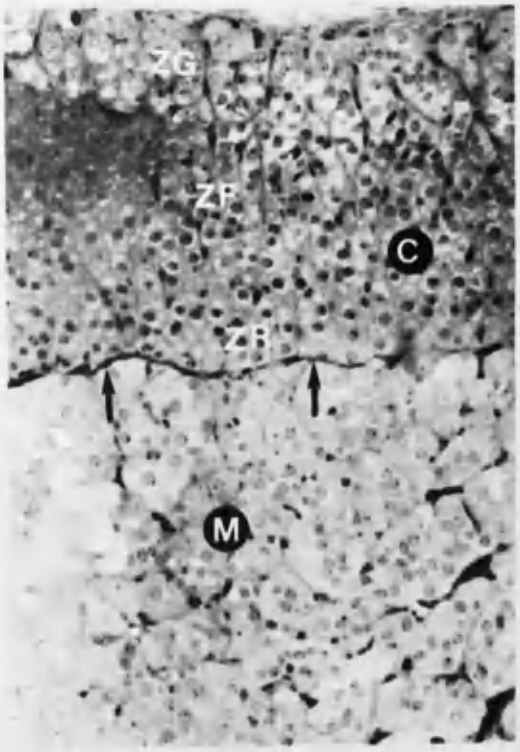


Fig. 3.14.

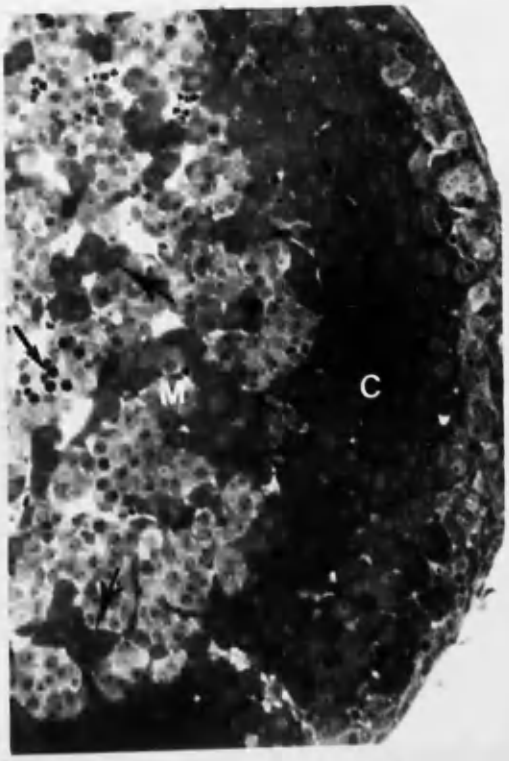


Fig. 3.15.

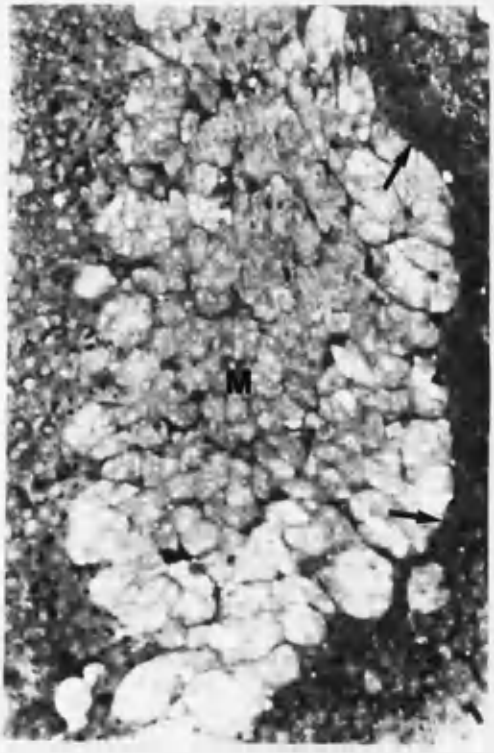
Fig. 3.16. Transverse section through 1 wpm adrenal gland showing chromaffin cells (M) assuming their central position with some cells arranged in cords. Light and dark chromaffin cells are obvious here. It also shows the developing X-zone (arrows). (Bar=100 μm).

Fig. 3.17. Transverse section through 2 wpm showing the medulla of the adrenal gland with most chromaffin cells in cords. Two types of chromaffin cells, light (small arrows) and dark (large arrows) are quite obvious. (Bar=50 μm).

Fig. 3.18. Transverse section through 1 dpm adrenal gland showing two types of chromaffin cells,light (small arrow) and dark (large arrows). It shows also small cells (small white arrow heads) and cortical cells distinguished by their stain (large white arrow heads). (Bar=25 μm).

All of the above figures are of araldite semithin sections stained with toluidine blue.

dpm= day postnatal mouse, M=medulla, wpm= week postnatal mouse



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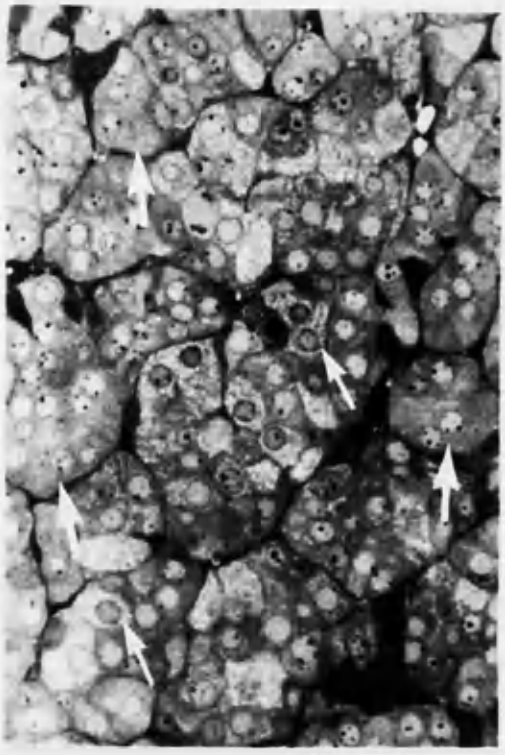

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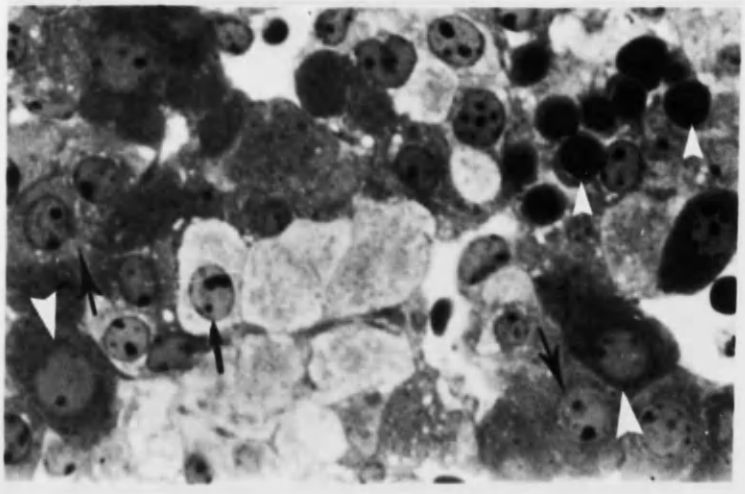


Fig.3.18.

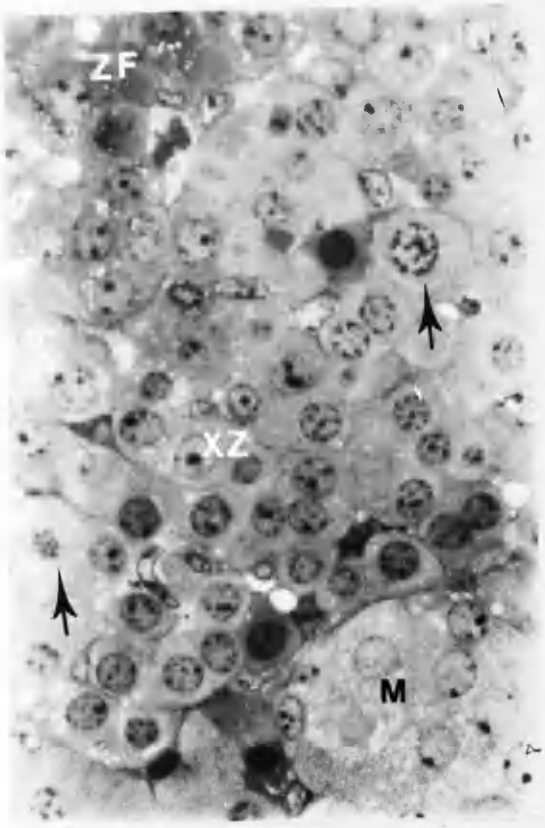
Fig. 3.19. Transverse section through 2 wpm adrenal gland showing mitotic figures (arrows) among the cells of the X-zone (XZ). (Bar=25 μm).

Fig. 3.20. Transverse section through the adrenal of an adult 16 day pregnant female mouse showing the cortex (C), medulla (M) and the the X-zone (XZ). (Bar=100 μm).

Fig. 3.21. Transverse section through 3 wpm adrenal gland showing degenerating cells (arrow heads) within the X-zone (XZ). (Bar=25 μm).

All of the above figures are of araldite semithin sections stained with toluidine blue.

C=cortex, M=medulla, wpm=week postnatal mouse, XZ=X-zone



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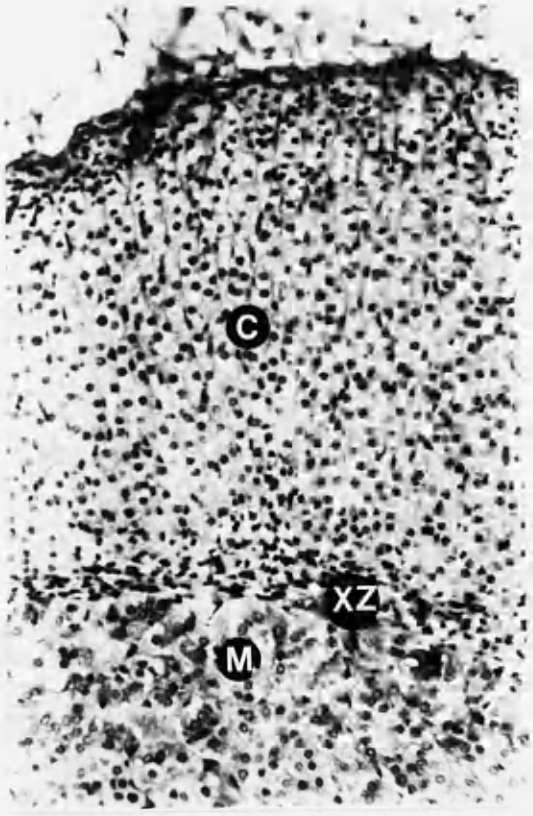

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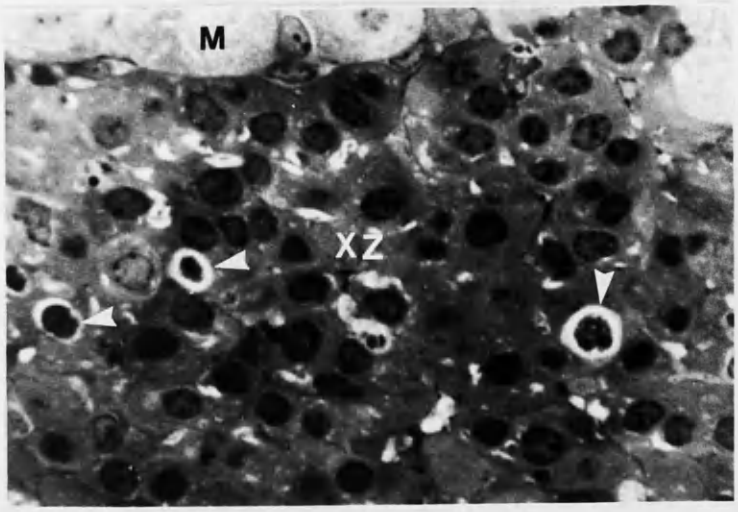


Fig.3.21.

Table 3.1 Outer Capsule

Age	Capsule width (μm)		No. counted
	mean	range	
15 dme	15.6	17.7-12.9	3
17 dme	13.6	15.5-11.8	3
1 wpm	16.3	18.3-13.95	4
2 wpm	17.0	18.5-15.6	3
3 wpm	17.1	18.3-15.3	3
5 wpm	18.8	21.7-16.1	3

dme = day mouse embryo
wpm = week postnatal mouse

Table 3.2 X-zone Measurement

Age	X-zone width (μm)		No. counted
	mean	range	
1 wpm	17.1	22.4-8.3	3
2 wpm	40.9	43.1-37.5	3
3 wpm	74.8	80.6-70	3

Fig. 3.22. Electron micrograph of 14 dme adrenal showing chromaffin cell (M) and cortical cell (C). Arrows represent microtubules within the cortical cell. Arrow heads represent chromaffin granules. (Bar=0.64 μm).

Fig. 3.23. Electron micrograph of 16 dme showing ultrastructure of a cortical cell. Rough endoplasmic reticulum (arrows), lipid droplet (lp), and mitochondria (m). (Bar=0.61 μm).

C=cortical cell, M=chromaffin cell, lp=lipid droplet m=mitochondria, n=nucleus

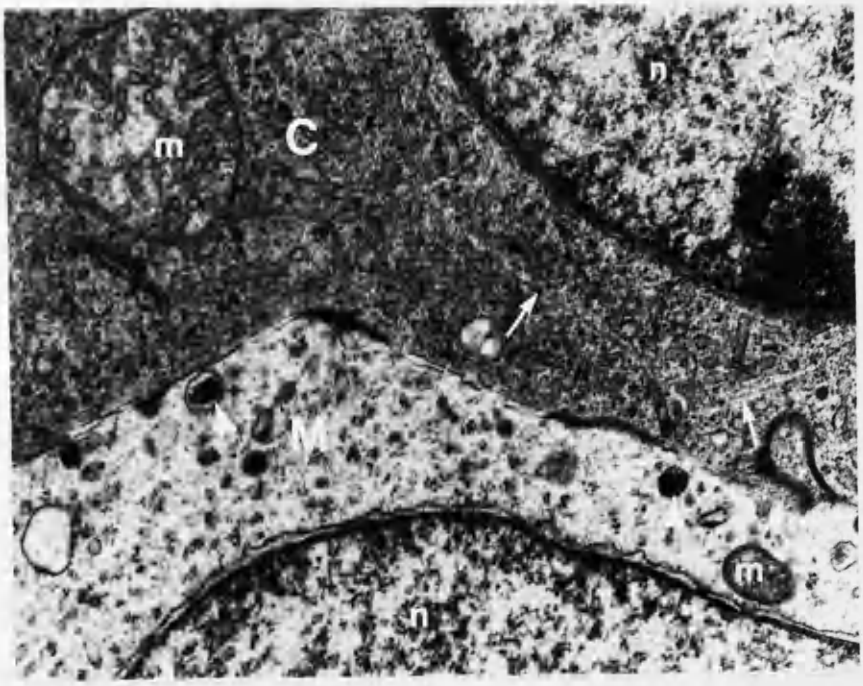


Fig. 3.22.

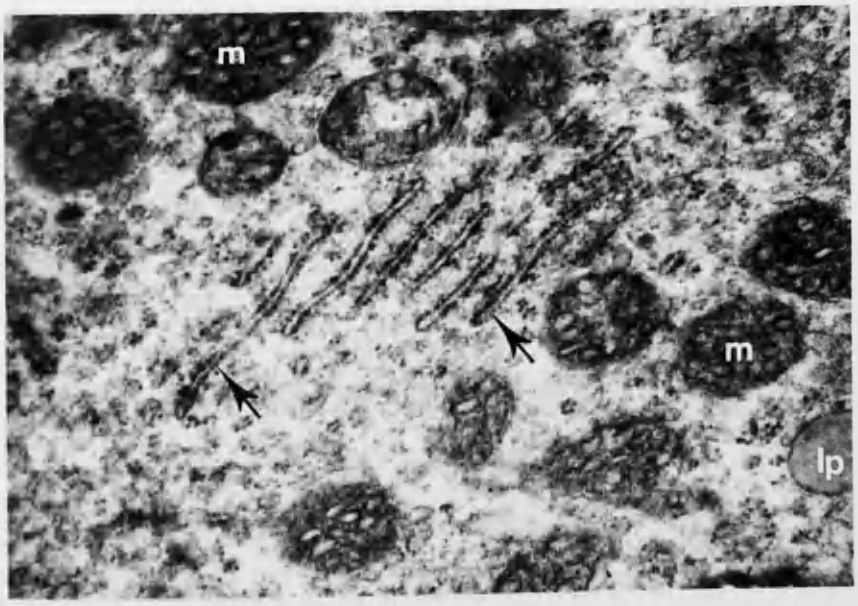
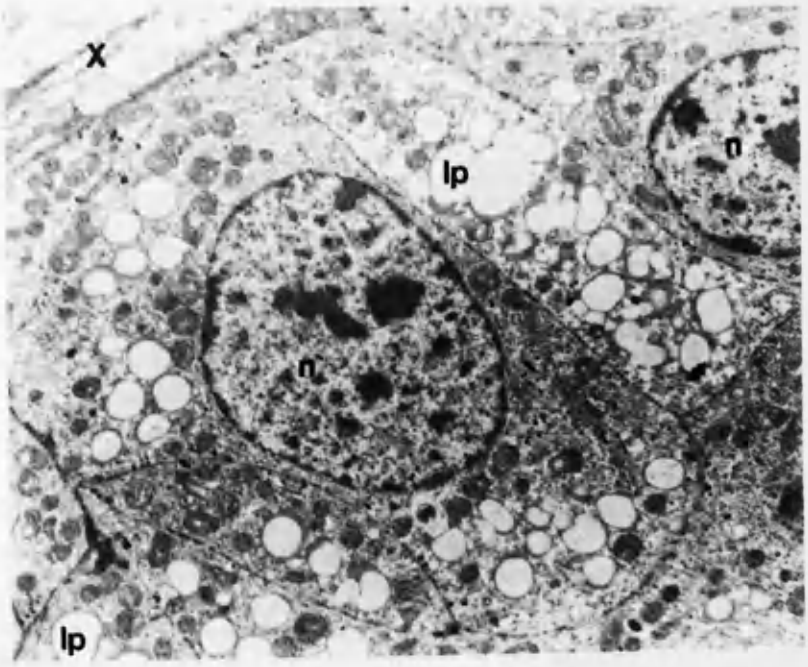


Fig. 3.23.

Fig. 3.24. Electron micrograph of 2 wpm zona glomerulosa cells with large number of lipid droplets (lp). (Bar=3.08 µm).

Fig. 3.25. Electron micrograph showing chromaffin cells of 14 dme showing some chromaffin granules attached to cell membrane (arrows). (Bar=0.53 µm).

dme=day mouse embryo, wpm=week postnatal mouse, n=nucleus, X=gland's capsule



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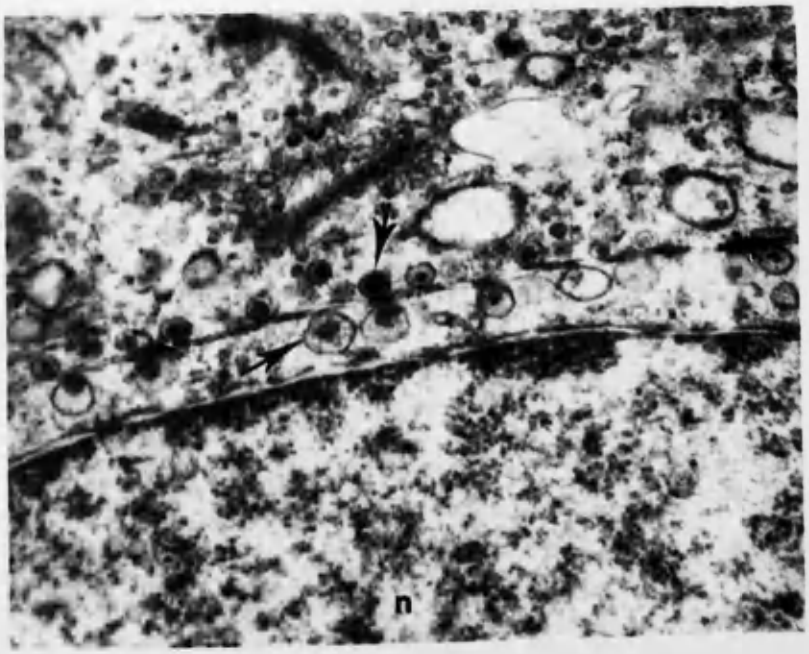
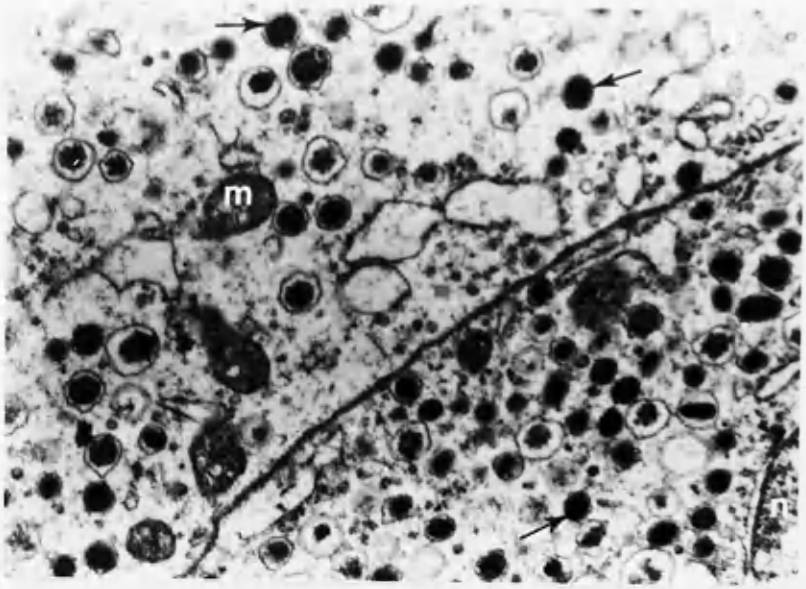


Fig. 3.25.

Fig. 3.26. Electron micrograph of 2 wpm showing two chromaffin cells with mainly electron dense granules (small arrows). The two cell membranes are closely apposed with a very narrow, even gap between (compared with fig. 3.30). This may represent a gap junction (Bar=0.81 μm).

Fig. 3.27. Electron micrograph of 2 wpm adrenal showing microtubules within chromaffin cells (arrows). Two types of chromaffin granules are present. The large arrow head points to (NA) and the small arrow head points to (N) granules. (Bar=0.53μm).

m=mitochondria, n=nucleus, wpm=week postnatal mouse



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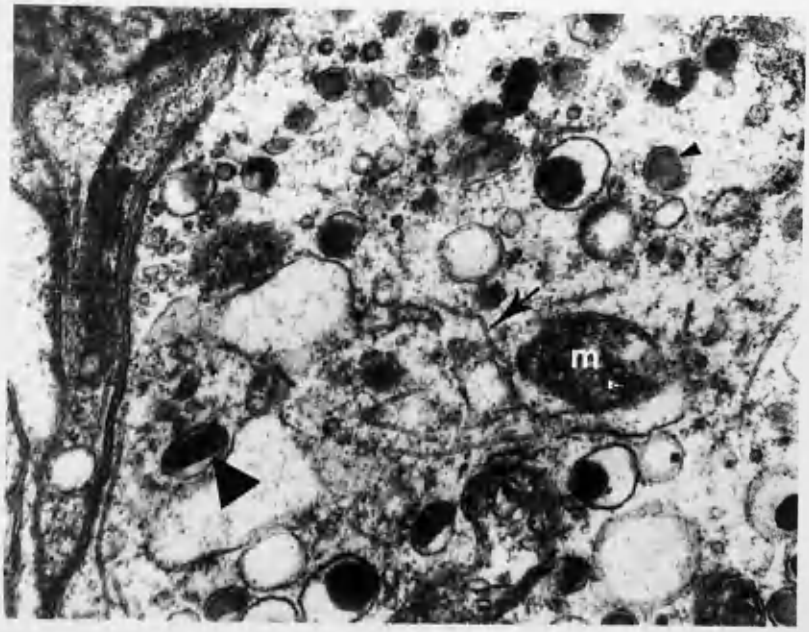


Fig.3.27.

Fig. 3.28. Electron micrograph of 14 dme showing the ultrastructure of a chromaffin cell; Golgi complex (large arrow), microtubules (small arrows), mitochondria (m) and dilated rough endoplasmic reticulum (rer). (Bar=0.81 μm).

Fig. 3.29. Electron micrograph of 2 dpm showing an usual "small cell" (SC) between cortical (left and medullary (right) cells. This cell appears to be binucleated. The cytoplasm is densely stained and has an unhealthy appearance, characteristic of these "small cells". (Bar=1.41 μm).

dme=day mouse embryo, dpm=day postnatal mouse, m=mitochondria, n=nucleus, rer=rough endoplasmic reticulum, SC=small cell

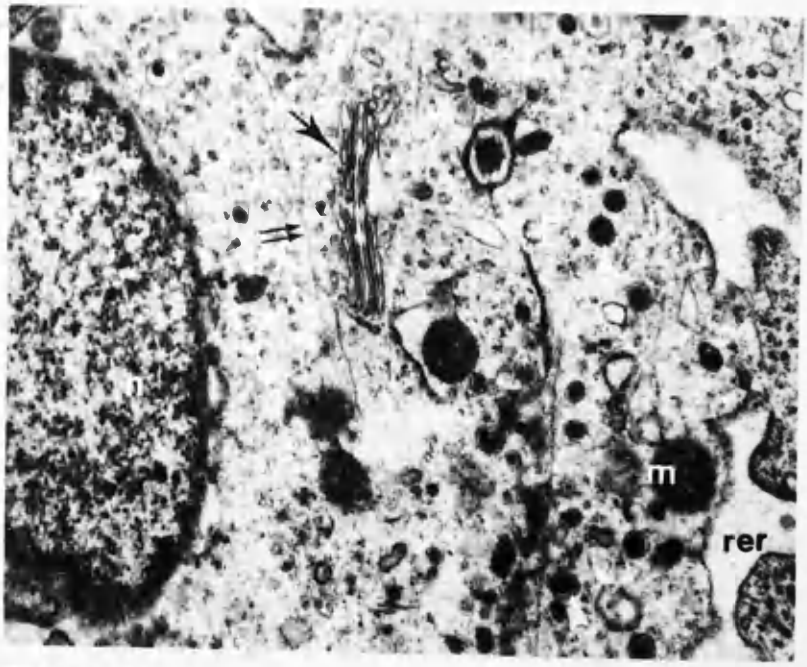


Fig.3.28.

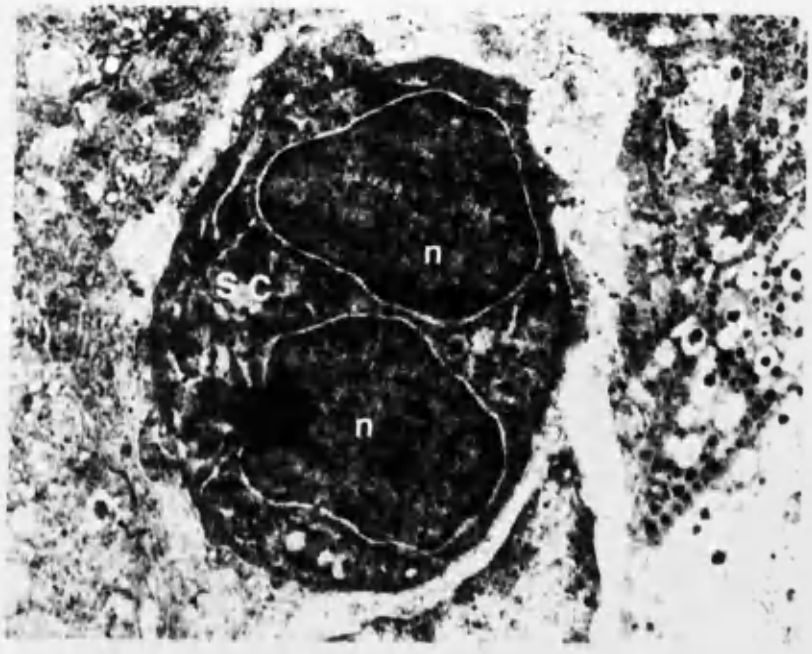


Fig.3.29.

Fig. 3.30. Electron micrograph of 15 dme showing junctions between two cortical cells. The large arrows indicate possible small desmosomes, and the small arrows point to areas of close membrane opposition, possibly gap junctions. (Bar=0.56 μm).

Fig. 3.31. Electron micrograph of 18 dme chromaffin cell showing microtubules (large arrows), microfilaments (small arrows). Arrow heads point to chromaffin granules. (Bar=0.31 μm).

dme=day mouse embryo, m=mitochondria

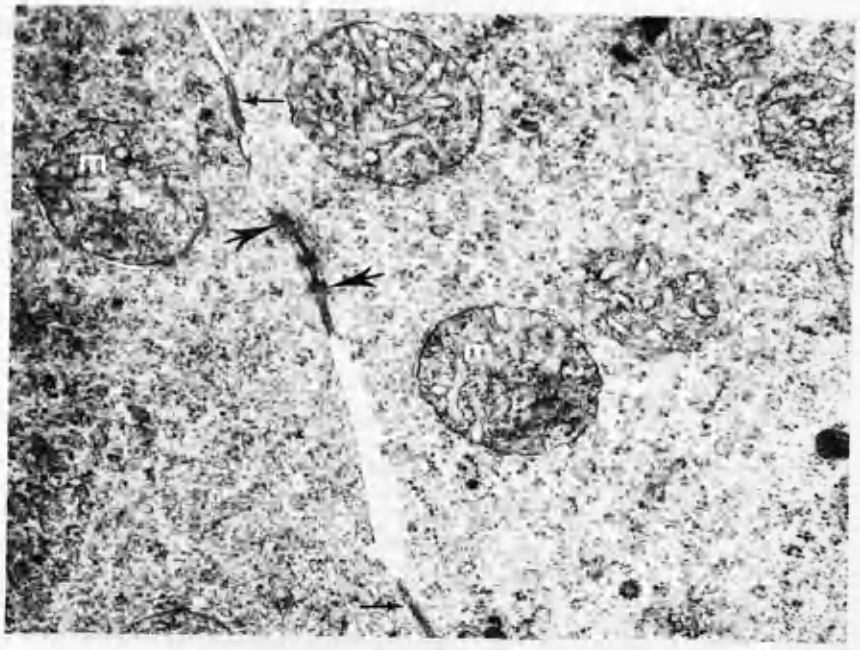


Fig. 3.30.

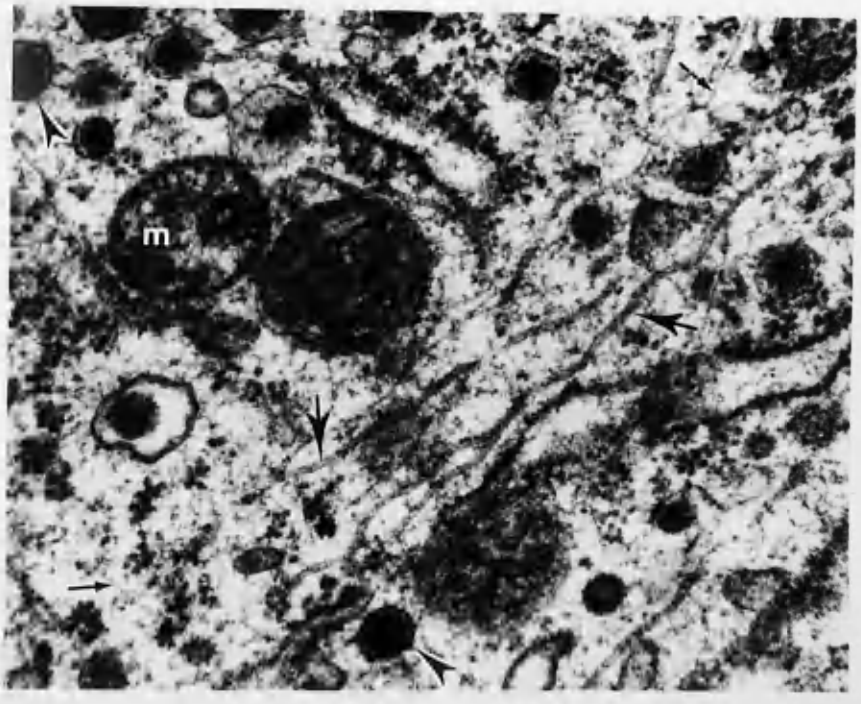


Fig. 3.31.

Fig. 3.32. Electron micrograph of 16 dme showing dying cells of both chromaffin (dmc) and cortical (dcc) types. (Bar=6.71 μm).

Fig. 3.33. Electron micrograph of 18 dme showing dying cortical cell (dcc). (Bar=3.08 μm).

cc=cortical cells, dcc=dying cortical cells, dmc=dying chromaffin cells, dme=day mouse embryo, mc=chromaffin cells, n=nucleus

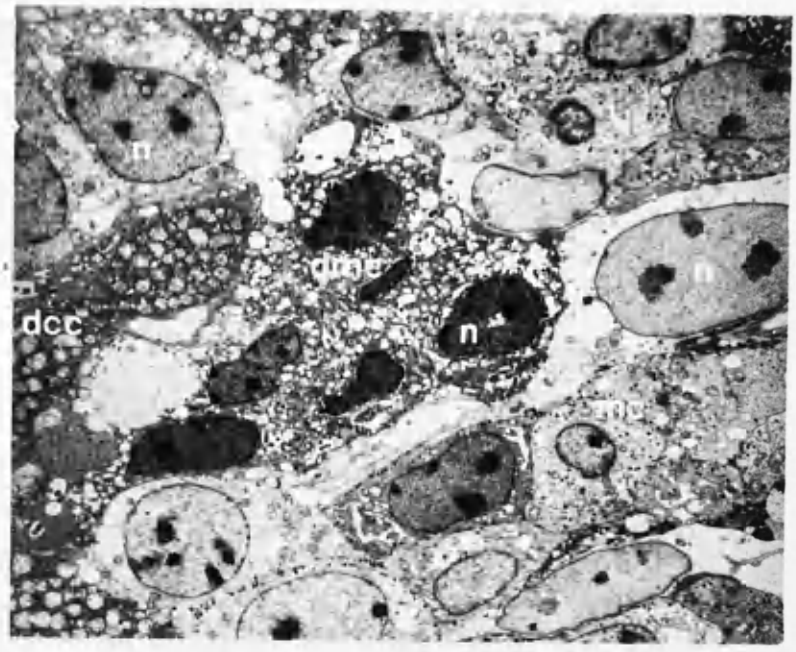


Fig.3.32.

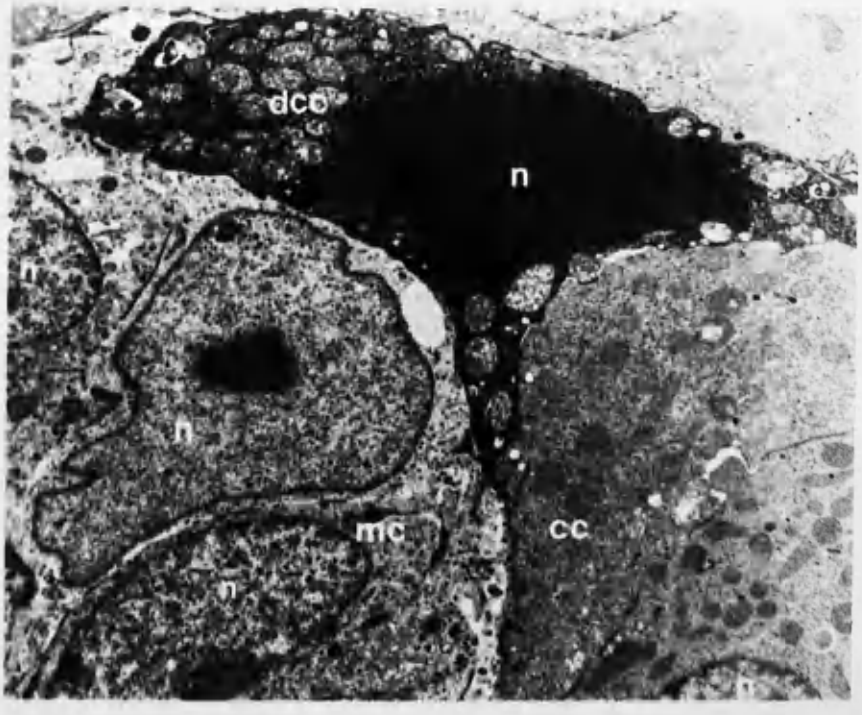


Fig.3.33.

Fig. 3.34. Electron micrograph of 1 wpm showing dying cortical cell (arrows). (Bar=3.08 μm).

Fig. 3.35. Electron micrograph of 3 wpm showing the characteristics of the degenerating X-zone with dark nuclei (n) and bizarre appearance of mitochondria (arrow heads). (Bar=3.08 μm).

C=cortical cells, M=mitochondria, n=nucleus, wpm=week postnatal mouse

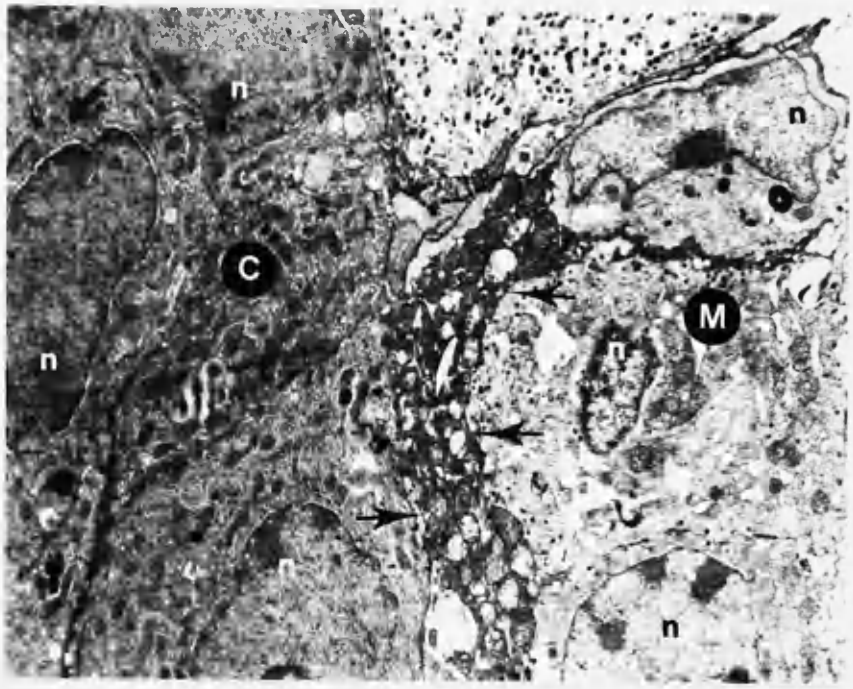


Fig. 3.34.

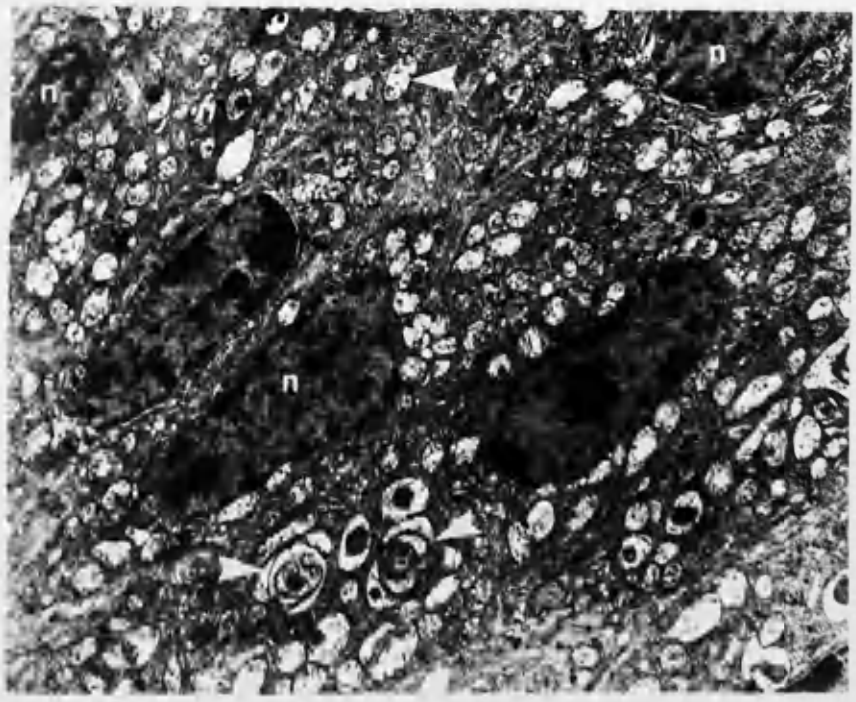


Fig. 3.35.

Fig. 3.36. Electron micrograph of 35 dpm showing the inner capsule (arrows) separating the cortex (C) from the medulla (M). (Bar=1.41 μm).

Fig. 3.37. Scanning electron micrograph of fractured 15 dme adrenal showing a blood vessel (bv) and blood cells (arrow heads). (Bar=19.6 μm).

dme=day mouse embryo, dpm=day postnatal mouse

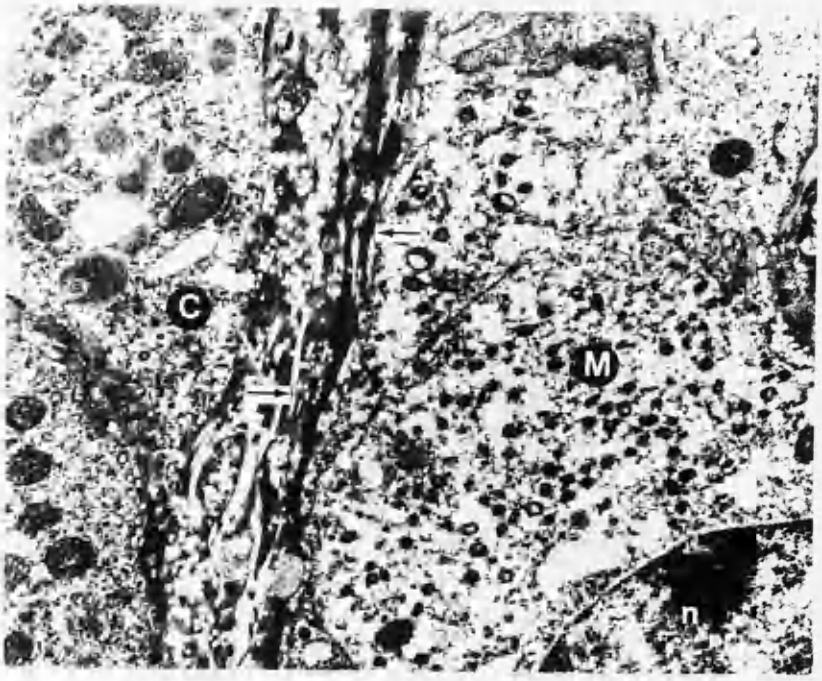


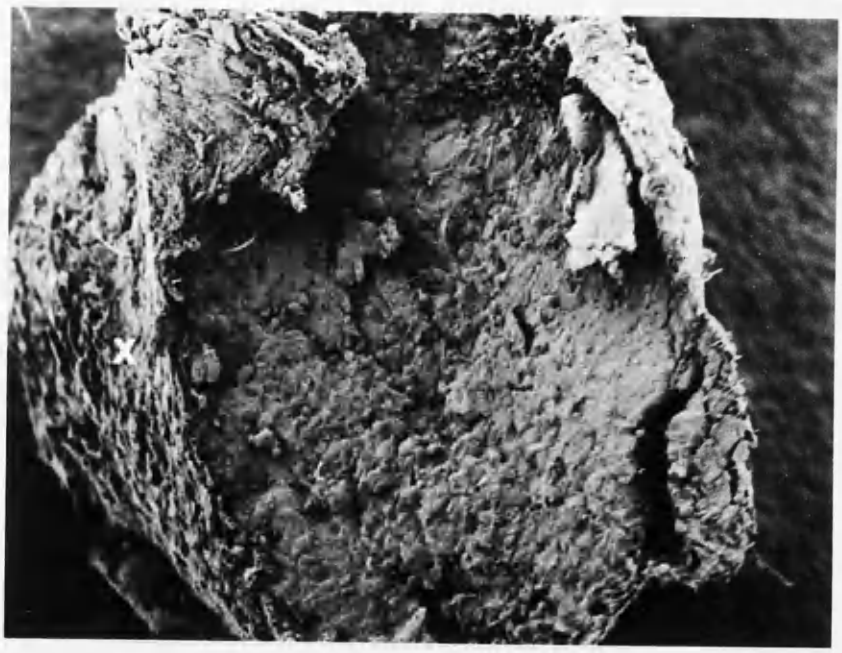
Fig. 3.36.

Fig. 3.37.

Fig. 3.38. Scanning electron micrograph (SEM) of fractured 15 dme showing how adrenal tissues look with SEM. (Bar=78.4 μm).

Fig. 3.39. Scanning electron micrograph of fractured adult adrenal showing the inner capsule (Ic) separating the cortex (C) from the medulla (M). (Bar=19.6 μm).

C=cortex, dme=day mouse embryo, Ic=inner capsule, M=medulla, X=gland's capsule



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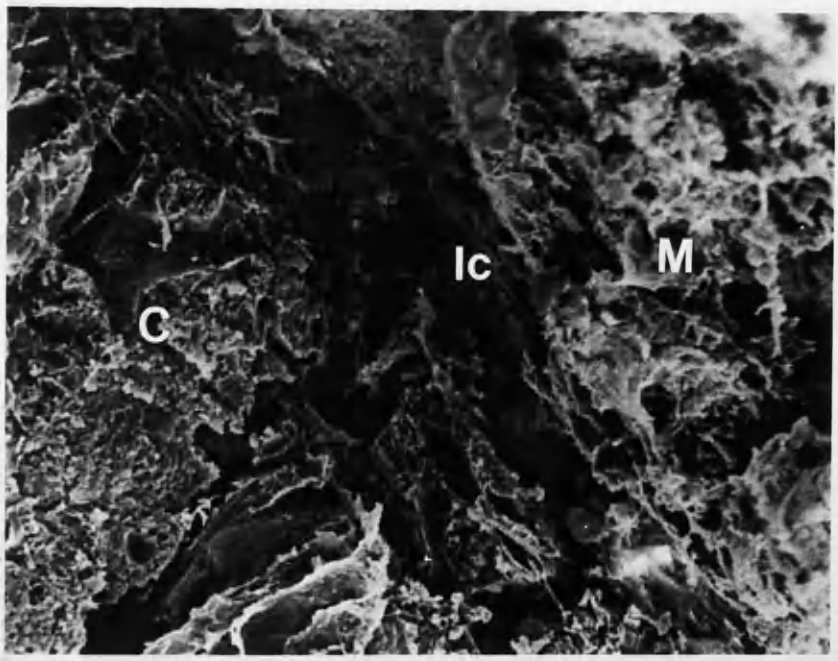


Fig. 3.39.

3.3 Discussion

There has not been a fully detailed study on the development of the mammalian adrenal gland as a whole since the work of Waring (1935), who studied the development of the mouse adrenal gland both during embryonic and postnatal life, at light microscope resolution. There have been several more recent studies on individual aspects of mammalian adrenal development at light and transmission electron microscope levels, but these have concentrated on cell differentiation rather than morphogenesis, and have not always agreed in detail.

This study is intended to give a detailed description of mouse adrenal gland development both in embryonic and postnatal life in order to clear up discrepancies in the literature, and to concentrate on morphogenetic aspects.

The development of the mouse adrenal gland takes place during embryonic life and goes on for several weeks after birth, that is when the gland has achieved the adult characteristics. This is shown by the presence of of two distinct regions, the cortex and medulla. The cortex possesses three zones: the zona glomerulosa, fasciculata and reticularis, and the medulla has two types of chromaffin cells, the adrenaline and noradrenaline cell types. An **inner capsule** is formed between the cortex and the medulla as a result of X-zone degeneration.

3.3.1 Overall Organisation of the Gland

During early formation, the gland is a loose structure, but it becomes compact in late embryonic stages. The outer capsule is clearly marked by the 13th day of gestation. The capsule is loose in early stages of embryonic development but becomes compact in later stages.

Cortical and chromaffin tissues are intermingled during early development but start to sort out into inner and outer regions by the 16th day of gestation. By the end of the first week of postnatal life the two regions are distinct, medulla and cortex, and by the 5th week the adrenal has achieved its adult characteristics.

Cell death was observed during embryonic stages, though it is also present in postnatal life in the X-zone.

3.3.2 The Timing of Cortical Cell Zonation

Cortical tissue is intermingled with chromaffin tissue during early embryonic life. By 17 day, mouse embryo cortical cells start to show some degree of zonation with the appearance of the zona fasciculata. Waring (1935) stated that both the zona glomerulosa and fasciculata are present prior to birth, though not fully differentiated and that the outline of the future zona fasciculata starts to appear in the 16th day mouse embryo. In my specimens, the zona glomerulosa and zona fasciculata start to become clear on about the 1st day after birth and by the 14th day both zones have become well established. Deacon *et al* (1986) stated that the zona glomerulosa and fasciculata start to develop on about the 3rd day after birth and added that on about the 9th day after birth the zona fasciculata is well formed. These differences in the timing of zonation may be due to genetical variation, since the different studies use different strains of mice. Waring (1935) did not specify his mice strains; Deacon *et al* (1986) used the Swiss albino strain, and I used the Glasgow hybrid breed (a cross between the A2G/Tb, A/Tb, C57BL/Tb and GFF lines).

A careful examination of the illustrations in Waring (1935) and Deacon *et al* (1986) do not reveal any differences in the criteria used for judging zonation between their studies and mine. Their work was based on thick wax sections (6 μm), whereas mine is based on semithin araldite sections (1 μm), which I feel may give more reliable results on the precise timing of histogenetic events.

By the end of the 1st week after birth, a third zone becomes apparent; the X-zone. The X-zone is not one of the adult zones but rather a transitory zone which will degenerate in the next few weeks. The other main adult zone is the zona reticularis, which will develop later.

The zona reticularis is well developed in the 5th week male mouse adrenal gland. It starts to develop while the X-zone is undergoing degeneration. In my specimens, the zona reticularis starts to appear at around 32 days. Howard-Miller (1927) claimed that the zona reticularis starts to develop after the disappearance of the X-zone, whereas Waring (1935) argued that is appears at the time when the X-zone starts to disappear. With regards to the appearance of the zona reticularis the results of my study are in harmony with those of Waring (1935). Howard-Miller (1927) and Waring (1935) agreed that the zona reticularis cells differentiate from the inner cells of the zona fasciculata, whereas Müntener & Theiller (1974) claimed that the zona reticularis cells differentiate from the zone of "small cells" (3rd zone). Müntener & Theiller (1974) argued that the postnatal mouse adrenal possesses four zones (zona glomerulosa, fasciculata, zone of small cells and the X-zone). In my study, the results showed that zona reticularis cells differentiate from the outer layer of the X-zone which differentiates from the inner

part of the zona fasciculata. The "small cell" zone which had been claimed by Müntener & Theiller (1974) as the third zone in the adrenal cortex of the mouse was not seen in my specimens.

3.3.3 Chromaffin Tissue Organisation and Differentiation

Chromaffin tissue starts to to join up at the centre of the gland on about the 16th day of gestation, but many patches of chromaffin cells are still intermingled with cortical tissue outside the centre. Waring (1935) showed that most chromaffin cells are present in the centre of the gland by the 16th day of gestation.

The final centralisation of the chromaffin tissues takes place during the 1st week of postnatal life. After this time, I found no chromaffin cells outside the medullary region. To the best of my knowledge no one has commented previously on the timing of chromaffin cells entry into the medulla.

During early embryonic life most chromaffin cells show a high nuclear to cytoplasmic ratio. These cells were termed "immature" by Miller & Unsicker (1981). Mature chromaffin cells are characterised by a decrease of nuclear to cytoplasmic ratio (Miller & Unsicker, 1981). The mature cells start to appear by the end of embryonic life and become more obvious in neonatal life especially about the end of the 1st week after birth. They become differentiated into adrenaline (A) and noradrenaline (NA) cell types about this time. Jurecka *et al* (1978) indicated that the medullary cells of the mouse adrenal gland are fully differentiated on about the 3rd-4th week after birth; Müntener & Theiller (1974) stated the chromaffin cells of the adrenal are fully differentiated by the 3rd week after birth. On the other

hand, Coupland (1984) stated that some undifferentiated chromaffin cells (both A and NA granules in the same cell) are found in a 3-6 week old mouse and that a population of undifferentiated cells is retained until adult-hood. In my specimens, chromaffin cells started to differentiate into (NA) and (A) cell types by 1 day of postnatal life. Two types of chromaffin cells could be identified according to their stain by 1 day after birth. One type stained lighter than the other. At light microscope level, dark cells may be (NA) or undifferentiated chromaffin cells, whereas light cells are (A) chromaffin cells. By 2-3 weeks, most chromaffin cells had differentiated into (A) and (NA) cell types, but I did not check at TEM level whether any undifferentiated cells remained in later postnatal life. Previous studies on the beginning of chromaffin cells differentiation have been mainly in the rat. My results show that in mice, as in rats, chromaffin cells differentiation is first apparent in early postnatal life (Daikoku *et al*, 1977; El-Maghraby & Lever, 1980 and Verhofsted *et al*, 1985).

Waring (1935) found that chromaffin cells start to be arranged in cords on about the 14th day after birth and that this arrangement then persisted throughout the mouse's life. In my specimens, medullary cells started to be arranged in cords on about the 4th day after birth and by 14 days most chromaffin cells were already in cords, separated from each other by thin connective tissue layers. Again Waring (1935) made his observations on thick wax sections (6 μm) whereas mine were on semithin sections (1 μm), and this along with possible genetical differences could account for these differences in timing.

3.3.4 X-zone and Inner Capsule

The X-zone is a transitory layer of cells appearing in postnatal life of mice and disappearing by the time of puberty in males and first pregnancy in females.

All previous workers have agreed that the X-zone develops in postnatal life within the first 10 days after birth, except Waring (1935). Waring (1935) claimed that the X-zone cells develop during late embryonic stages as an "interlocking zone" an area of cells around the central region, interdigitating with the accumulating chromaffin tissue. He called the darkly stained embryonic cortical cells "X-zone cells" and went on to say that more of these cells accumulate around the central region in postnatal stages to develop a distinct zone by 8 days of postnatal life. Waring (1935) made no comment on the region of these cells other than that they were cortical cells.

My results disagree with those of Waring (1935) with respect to the X-zone's origin and are in harmony with those of Hirokawa & Ishikawa (1974) who indicated that the X-zone cells develop after birth. My findings agree with the conclusion of Hirokawa & Ishikawa (1974) who argued that cells of the X-zone differentiate from the inner cortical cells especially those of the zona fasciculata.

Howard-Miller (1927) showed that the X-zone is obvious on the 10-14th day after birth. Waring (1935) argued that X-zone cells are present as early as day 1 after birth, intermingling with the other adrenal gland tissues (cortical and chromaffin), and form an obvious zone by the 8th day after birth. Hirokawa & Ishikawa (1974) claimed that the X-zone starts its development on the 8th day after birth in both males and females and becomes an obvious layer on the 10-14th day. Müntener & Theiller (1974)

confirmed that the X-zone can be identified quite clearly between the 2-3rd week after birth. Deacon *et al* (1986) recently described the X-zone as present as a distinct narrow band from about the 9th day after birth. In my specimens, cells of the X-zone cells were present as early as day 4 after birth scattering at the interface of th medulla and cortex. The X-zone formed a thin layer of cells surrounding the medulla by the end of the first week.

Howard-Miller (1927) showed that the X-zone reaches its maximum size on the 3rd week after birth in both males and females, and Waring (1935) argued that the X-zone reaches its maximum size between the 18-25 day after birth. Hirokawa & Ishikawa (1974) indicated that the female X-zone continued its development until the age of 28-35 day after birth. Holmes & Dickson (1971) agreed with Howard-Miller's (1927) observations. In my specimens the X-zone grows to reach its maximum size after 21 days.

Previous reports have shown some variability in the timing of X-zone disappearance in males. Hirokawa & Ishikawa (1974) found very rapid degeneration with the X-zone gone by 30 days. Howard-Miller (1927) showed that X-zone disappeared about 40 days in males. In my specimens, the X-zone disappeared by 35 days. In females, the X-zone persists until first pregnancy.

It is clear from the above that the detailed timing of X-zone development and degeneration is somewhat variable, presumably due to genetical differences between mouse strains.

As a result of X-zone degeneration, an **inner capsule** is formed separating the medulla from the cortex, at about 35 days in males. The use of TEM and SEM revealed the structure of this

capsule. The inner capsule is made up of connective tissue fibres and the debris of dead cells. These results are in agreement with those of Waring (1935); Holmes & Dickson (1974); Hirokawa & Ishikawa (1974) and Deacon *et al* (1986).

No one previously has reported the pattern of growth and degeneration of the X-zone. I have found that cell death in the X-zone starts at the medullary side. While this is happening, the X-zone is still growing, shown by the presence of mitotic figures within the X-zone, mainly at the cortical side. The X-zone approximately double in width each week from 1-3 week postnatal. It is possible that this growth involves not only cell division but also cell transformation from the adjoining zona fasciculata, but it cannot involve distant immigration, since no cells of X-zone morphology are seen at a distance from the X-zone. To determine whether both cell transformation and cell proliferation contribute to X-zone growth, a detailed morphometric analysis of the early postnatal adrenal would be necessary.

Hirokawa & Ishikawa (1974) indicated that mitosis was often found within the X-zone but made no comment on the pattern of X-zone growth.

3.3.5 Morphogenetic Features

There have been very few previous descriptions of intercellular junctions in the developing adrenal gland. Decker (1981) stated that gap junctions are present between cortical cells of the adrenal gland of the mouse, rat and rabbit embryos. Joseph *et al* (1973) found that gap junctions and desmosomes between cortical cells in fetal rabbits. Black (1972) showed that desmosomes-like junctions occur between cortical cells of the fetal guinea pig. In postnatal rat adrenals, Placias (1979)

showed that desmosomes and gap junctions are present between cells in all zones of the cortex but that tight junctions are present only in the zona glomerulosa and only after 2 weeks. In adult rat adrenals, desmosomes are present between chromaffin cells (Coupland, 1965a) and desmosomes and gap junctions are present between the cortical cells (Friend & Gilula, 1972).

In my study, gap junctions and desmosomes were present between like and unlike cells of the adrenal gland (chromaffin and cortical) and showed no obvious changes in all the stages studied (embryonic and postnatal).

There have been no previous studies on cells shape, intercellular spaces, or microtubule (MT) and microfilament (MF) distribution in the embryonic and early postnatal mouse adrenal. Black (1972) on fetal guinea pig; and Friend & Gilula (1972) on adult rats, showed that intercellular spaces are present between cortical cells. In my study intercellular spaces within cortical tissue and chromaffin patches were remarkably straight and even at all stages. This was true also for intercellular spaces between unlike cells; that is cortical-chromaffin interconnections. Although early mouse embryonic adrenal glands were less compact, quite wide spaces between cells of same group were present but not between intra-groups. Those intercellular spaces were smooth and straight. No previous observations were recorded on this.

In both cortical and chromaffin tissues, the cell surface was predominantly smooth and straight at all stages of development studied. Although convoluted interdigitating cell surfaces occasionally occurred, there was no sign of elongated cellular processes.

MT and MF were present within the embryonic adrenal cells and seemed more abundant in chromaffin cells than in corticals. This difference in MT and MF presence may, however, have been due to differences in cytoplasmic density, making it easier to see MT and MF in the less dense chromaffin cell cytoplasm. The presence of MT and Mf may be an indication of active cell motility, but since they are found in some cells after gland tissues segregation has been completed, this evidence is not very strong.

3.3.6 Cell Death During Embryonic Life

During embryonic development, I found a class of cells which stained very dark (darker than the rest of all the embryonic adrenal tissues) with toluidine blue. This type of cells was observed in all embryonic stages studied (14-18 day mouse embryos). This type of cells were distributed throughout the gland both around the centre and at the periphery. They were characterised by the presence of vacuoles, shrinkage of the nuclei and by their irregular shape. All these characteristics suggested that these were cells undergoing death. TEM observations confirmed that these cells were of both chromaffin and cortical types.

Widely distributed cell death in the embryonic adrenal has not been previously reported. The only previous reports of cell death in the adrenal concern the death of the X-zone cells during inner capsule formation (see section 3.3.4 above). The occurrence of embryonic cell death in the adrenal will be discussed in chapter 8.

CHAPTER FOUR

Development of the Chick Adrenal Gland

4.1 Introduction

4.1.1 Adult Adrenal Gland Structure in Birds

The avian adrenal glands are paired organs which are part of the endocrine system. They lie on the anterior side of the kidneys, one on each side. The adrenal gland consists of two distinct tissues, the cortical and the chromaffin tissue. The two tissues are intermingled with each other as patches dispersed throughout the gland, there is no cortical-medullary arrangement of the kind found in mammals. The avian cortical cells are arranged in cords, not as zones, and the chromaffin cells occur as groups or as single cells scattered among the cortical cords.

There is some debate over the distribution of cortical and chromaffin tissues in the avian adrenals. This will be covered in the following sections.

The pattern of adrenal gland tissue distribution throughout the gland depends mainly on the amount of each tissue present within the gland and also on the species.

The relative size of the adrenal gland varies with respect to the environment inhabitated by birds. Holmes & Phillips (1976) reported that marine birds have larger adrenals than those of fresh water, whereas brackish water birds have an intermediate size. They relate the difference in adrenal sizes to salt consumption.

Unsicker (1973d) claimed that the cortico-chromaffin ratio of the domestic adult fowl is 1:1. Siller *et al* (1975) showed

that the female fowl has a higher cortico-chromaffin ratio than that of the male.

4.1.1.1 Chromaffin Tissue

Arrangement of chromaffin tissues in the avian adrenal is sometimes described as being random, but in a comparative study of adrenals from many species of birds Hartman *et al* (1947) and Hartman & Albertin (1951) found that the pattern of chromaffin cell patches was characteristic for each species. The patterns related to the relative amount of chromaffin and cortical tissues. If the chromaffin tissue was present in small amount it tended to form distinct islets scattered throughout the gland. If in larger amount, then it was arranged in large elongated islets which might show some anastomosis. If chromaffin tissue was present in even larger amount, then it was arranged both in the interior and at the periphery, mainly at the surface of the gland.

In the pelican, chromaffin cells are confined mainly to the interior of the gland and occur as small irregular islets. In the Family Paridae, the adrenal gland has a relatively large amount of chromaffin tissue, arranged in large often elongated islets but with little anastomosis. In woodpeckers, chromaffin tissue is not in unusually large amount, but is found mainly at the periphery. In fowl and pigeon, chromaffin cells are present in groups found mainly in the interior of the adrenal. In quails, the chromaffin cells are present in large amount as large anastomosing islets both in the interior and at the periphery of the gland.

4.1.1.2 Cortical Tissue

Although avian adrenal cortical tissues do not show the obvious zonation of mammals, some workers believe that they do possess two zones: a narrow peripheral zone and a much wider central zone. Sivaram (1964, 1965) claimed that the two cortical zones are present in the 15 day chick embryo, but Hall & Hughes (1970) working on chick embryos (10-18 day) failed to confirm their presence. Fujita (1961); Kjaerheim & Kondics (1966); Kondics & Kjaerheim (1966); Kjaerheim (1968) and Aire (1980) on the young and adult fowl; Sheridan *et al* (1963) on the adult brown pelican; Haack *et al* (1972) on the adult duck and Unsicker (1973c) on several adult avian species; including domestic fowl, goose and pigeon, Pecking duck, gulls and house sparrow, gave evidence for the presence of two cortical zones in those species that they studied. Cronshaw *et al* (1974) and Pearce *et al* (1978) studied the adrenal gland of adult and young ducks but could not find the two zones under normal conditions at the light microscopic level, but did show the presence of the two zones at the electron microscopic level when the animals were exposed to high and low levels of corticotropic stimulation or when the adenohypophysis was removed.

The above workers who are in favour of avian cortical zonation, claimed that the two zones, peripheral and central zones, perhaps correspond to the zona glomerulosa and zona fasciculata of the mammalian adrenal cortex, respectively.

According to Sivaram (1965) the two cortical zones differ in their histological and cytological appearance. Sivaram (1965); Kjaerheim (1968); Unsicker (1973c) and Pearce *et al* (1978) showed that the two cortical zones differ in their ultrastructure. According to Kjaerheim (1968); Unsicker (1973c)

and Pearce *et al* (1978), the peripheral zone has cells which have mitochondria with elongated cristae and are rich in rough endoplasmic reticulum. In contrast, the central zone cells have mitochondria with round tubular cristae and are rich in smooth endoplasmic reticulum. Unsicker (1973c) claimed that the peripheral zone cells have low lipid contents whereas the central zone cells have high lipid contents. On the other hand, Sivaram (1964, 1965) showed that the peripheral zone cells have greater abundance of mitochondria and ascorbic acid, hence show higher metabolic and perhaps synthetic activity than those in the central zone.

On the other hand, Fujita (1961), in the domestic fowl, and Sheridan *et al* (1963), in brown pelican, claimed that cortical cells are the same, whether they are present at the centre or at the periphery of the gland. They found no ultrastructural differences.

Avian adrenal gland tissues -cortical and chromaffin- have cholinergic innervation. Unsicker (1973b) showed that chromaffin cells are innervated by preganglionic sympathetic nerve fibres which originate from the splanchnic nerves.

4.1.2 Development of the Chick Adrenal Gland

The origin, initial appearance and formation of the chick adrenal gland have been discussed in sections 1.4.1, 1.4.1.2, 1.4.2, 1.4.2.2 of chapter 1.

The morphogenesis of the chick adrenal gland takes place in early embryonic life. Dawson (1953) claimed that the final organisation of the adrenal tissues is completed by the 14th day of incubation. By then both cortical and chromaffin tissues are

present and intermingled with each other. The cortical tissue is arranged in cords whereas the chromaffin tissue is seen as single cells or as in groups of cells.

The adrenal tissues start to synthesis and secrete hormones very early in embryonic life. The cortical tissue starts to function on about the 10th day of incubation, whereas the chromaffin tissue starts on about the 11th day of incubation, secreting mainly noradrenaline (NA) (Sivaram, 1964). Mastrolia & Manelli (1969) showed that the (NA) granules are the first to appear in the chromaffin cells. They also found adrenaline (A) and noradrenaline (NA) granules present in the same chromaffin cells on the 13-15th day of incubation and that by the 15th day, separate (A) and (NA) cell types are identifiable. Hall (1970) and Hall & Hughes (1970) found that (A) and (NA) granules are present in the same cell prior to the 17th day of incubation when separate (A) and (NA) cell types can be identified. On the other hand, Sivaram (1965) and Mezhnin (1969) claimed that the (A) and (NA) granules are present in the same chromaffin cells during embryonic life and become differentiated into (A) and (NA) cell type only several weeks after hatching.

During embryonic development, mitotic activity is present in both cortical and chromaffin tissues. According to Romanoff (1960) mitotic activity is more within the cortical than the chromaffin tissues. Hall (1970) showed that mitotic activity reaches its maximum within the cortical tissue of the chick on about the 14th day of incubation. The rate of cortical tissue growth in embryonic life is higher than that in the chromaffin tissue (Sivaram, 1965 and Hall, 1970). Sivaram (1965) showed that the peripheral zone of the young and adult chick adrenal cortical tissue is mitotically active, whereas the central zone shows a

degenerative state. He suggested that cortical cells migrate internally from the periphery to replace the dying cortical cells of the central zone. In contrast, Haack *et al* (1972) found that the central zone of the young and adult duck adrenal cortical tissue is mitotically active and suggested that cortical cells migrate outward from the central zone toward the periphery. Sivaram (1965) also showed that by the end of the first few weeks after hatching in the domestic fowl, the chromaffin tissue increases and its proportion of the gland becomes higher than the cortical tissue and stays in that state throughout the rest of the animal's life.

4.1.3 The Aims of This Study

The main aim of this part of my work is to compare morphogenetic features of chick adrenal development with mouse. I have examined the internal structure of the chick adrenal gland in later stages of development (15-19 day embryos i.e. Hamburger & Hamilton stages 41-45; and 10 day post-hatching), using light and electron microscopy. These stages were studied as a comparison with the stages when in the mouse, chromaffin cell patches are joining up to form a central mass. I hoped that by looking at the ultrastructure of chick adrenal cells, I might get some clues as to why a central medulla does not form in the chick. An additional general aim is to amplify previous workers accounts on chick adrenal morphogenesis, especially on cell death, and at the TEM level, where previous work is rather limited.

4.2 Results

4.2.1 Macroscopic Observations (fig 4.1)

The adrenal glands of the chick embryo lie dorsal to the anterior end of the developing urinogenital organs, just posterior to the lungs. The adrenals vary in size and in shape even in the same embryo. The right adrenal has a blunt-edge shape, pointed caudally, whereas the left adrenal is less constant in its shape and slightly longer than the right one. They are wholly or partly covered by the gonads, especially the left adrenal. The left gonad is ventral to the left adrenal gland and tends to obscure it, making it difficult to locate and dissect the left adrenal. On the other hand, the right adrenal is not covered by the right gonad and hence, its location and dissection is easier.

4.2.2 Microscopic Observations

4.2.2.1 Light Microscopy (LM)

Study of chick adrenal gland development was carried out on 15-19 day chick embryos (Hamburger & Hamilton stages 41-45) and on 10 day post-hatching chicks. By stage 41, migration of neural crest cells from outside the gland has already ceased.

In the semithin araldite sections (1 μm) I made, the cortical cells are stained lightly (light-blue) with toluidine blue and light brown with ammoniacal silver carbonate. In contrast, the chromaffin cells are stained darkly (blue-green) with toluidine blue and dark brown with ammoniacal silver carbonate.

It is important to point out that the staining reaction of chick adrenals was opposite to that of mouse adrenal tissues, where toluidine blue stained cortical tissue darkly, and

chromaffin tissue lightly, and ammoniacal silver carbonate stained cortical tissue darkly and chromaffin tissue lightly.

I have not been able to find any previous report of this difference, nor any explanation for it.

The description that follows is based on toluidine blue and ammoniacal silver carbonate stained sections.

4.2.2.1.1 Adrenal Tissues (15-19 day Chick Embryo & 10 day Post-Hatching)

Both chromaffin and cortical tissues are found to be intermingled in all stages studied (fig 4.2). During early embryonic stages the adrenal is loose but becomes compact in late embryonic stages. In post-hatching the adrenal has same features as that of late embryonic life.

The adrenal glands are very rich in blood supply. Different sizes of blood vessels are seen inside the gland and in its vicinity. There are no obvious changes with respect to blood supply during the stages studied.

4.2.2.1.1.1 Chromaffin Tissue

In the embryonic stages studied, the chromaffin cells are polygonal with almost round nuclei. They have a coarse cytoplasm. They are intermingled with the other adrenal tissues (fig 4.2). Chromaffin cells are found to be either as single cells (usually elongated in shape) or as groups of cells (cells are polygonal in shape) (fig 4.3). This arrangement was seen in all stages, though more cells are in groups and fewer as single cells near hatching. The chromaffin cells are distributed between cortical cords, and some chromaffin cells (singles or in groups) were seen

abutting blood vessels. Chromaffin cells (single and groups) are not uniformly arranged; they appear as randomly scattered patches, throughout the gland, including immediately under the capsule (fig 4.4).

By 10 days post-hatching the general organisation is similar to that of late embryonic stages (fig 4.5), but there are differences in detail, including an overall increased number of chromaffin cells, and also a great accumulation of chromaffin cells just beneath the capsule of the gland. Most chromaffin cells are now arranged in groups except for very few cells which are still seen as individuals. Single cells are elongated in shape, but cells in groups are polygonal.

In post-hatching, two types of chromaffin cells can be seen (fig 4.6); the difference becomes more obvious when ammoniacal silver carbonate stain is used, when cells are stained either very dark brown or light brown.

4.2.2.1.1.2 Cortical Tissue

The general appearance and characteristics of the cortical cells did not show any significant changes in all the embryonic stages (15-19 day of incubation; H&H stages 41-45) that were studied. The only obvious difference was an elongation of the cortical cords near the time of hatching. The cortical cells are arranged in solid double rowed cords which are surrounded by thin connective tissue layers and also by blood vessels (fig 4.3, 4.4). These cortical cords were seen throughout the gland. The cortical cells are polygonal with almost round nuclei. Lipid droplets are present within the cortical cells cytoplasm (fig 4.6).

Cortical cells stained lightly compared to chromaffin cells, but amongst the corticals are two classes of cells: a relatively dark and a very light cell when stained with toluidine blue (fig 4.7). The darkly stained cells form the majority, whereas the lightly stained cells are less frequent. Both cell types were present in the same cortical cord with no obvious difference in cell distribution in different parts of the gland. The two cell types were both present from the 15th day up to and including the 19th day of incubation (fig 4.7, 4.8).

By 10 days post-hatching, the general organisation of the cortical cells is similar to that of the late embryonic stages (fig 4.6), but there were differences in detail. By this time more cortical cells are arranged in larger cords and more cells exhibit more lipid droplets, represented by vacuoles within the cell cytoplasm (fig 4.6).

4.2.2.1.1.3 Sympathetic Ganglion Cells

Sympathetic cells are large and polygonal with large round to oval nuclei. They stained lighter than the two main tissues of the gland with both toluidine blue and ammoniacal silver carbonate stains.

Sympathetic ganglion cells were present in the centre and the periphery of the gland and also in the vicinity of the gland mainly around the capsule (fig 4.5, 4.8). Those sympathetic cells which are present within the gland seemed to have an association with the chromaffin cells (fig 4.8). The sympathetic ganglion cells which are located outside the gland's capsule are seen to be connected to those inside via nerve fibres (fig 4.5).

4.2.2.1.1.4 Mitoses

Mitotic figures were frequently seen in both cortical and chromaffin tissues. The mitotic figures were not confined to a specific area of the gland, but were found in all parts of the gland. No mitotic counts were made on chick adrenals.

4.2.2.1.1.5 The Adrenal Capsule

The capsule is made up of fibroblast cells and connective tissues including collagen fibres. During embryonic development, the adrenal gland capsule does not have a clear cut boundary, but interdigitates with other neighbouring tissues such as the urinogenital system and blood vessels (fig 4.4, 4.5). For this reason it was hard to make an accurate measurement of the capsule thickness. The maximum thickness that I could measure is 14.6 um for the 15 day chick embryo and 18.2 um for the 18 day chick embryo.

4.2.2.1.1.6 Cell Death

There is a class of cells stained very dark with darkly stained nuclei (fig 4.9). These cells are seen throughout the gland mostly as individuals but occasionally in small groups, and are located amongst both cortical and chromaffin patches (fig 4.9). I could not find these cells in 15 or 16 day embryos, but they occurred in the later stages. At the light microscopic level I could not identify which kind of cells these are, but they have the general appearance of dying cells.

4.2.2.2 Transmission Electron Microscopy (TEM)

The TEM work was carried out on 15-19 day chick embryos (41-45 H&H stages). Post-hatching stages were not examined at TEM level. It is not the objective of this study to give a detailed ultrastructural account of the adrenal gland. Such accounts are readily available elsewhere (Fujita, 1961; Hall & Hughes, 1970 and Ishimura & Fujita, 1981). After brief accounts of the main ultrastructural features of the main cell types, I will concentrate on the features of relevance to gland morphogenesis; cell junctions, presence of microtubules and microfilaments, and signs of cell death.

Almost all cortical and chromaffin cells have a polygonal shape with polygonal nuclei. The cortical and chromaffin cells differ in their ultrastructural features. The main features of the cortical cells are the abundance of mitochondria, the presence of smooth endoplasmic reticulum (SER) and the lipid droplets. In contrast, the chromaffin cells are characterised by the presence of chromaffin granules and rough endoplasmic reticulum (RER).

4.2.2.2.1 Chromaffin Tissue

There are two types of chromaffin cells, those with a high nuclear to cytoplasmic ratio and those with a high cytoplasmic to nuclear ratio, with the latter in the majority. The first group have few chromaffin granules and these granules are of the electron dense type. The second group contain both electron dense and moderately electron dense granules. The granules vary in shape, though most of them have an elongated shape and some have a spherical shape (fig 4.10). Both kinds of granules (electron dense and moderately dense) are present in the same chromaffin

cells during all the embryonic stages I examined (up to 19 days).

Chromaffin cells also have compact mitochondria, Golgi complex, RER and free ribosomes (fig 4.10).

4.2.2.2.2 Cortical Tissue

All cortical cells stain lightly compared to chromaffin cells, but amongst the corticals are two classes of cells; relatively dark and very light corticals (fig 4.11).

The light cells were seen in all embryonic tissues which were looked at. They are characterised by a clear cytoplasm and a scarcity of certain cytoplasmic organelles such as mitochondria, lipid droplets, SER, free ribosomes and also seem to lack a Golgi complex (fig 4.11).

The dark cells have the normal appearance of differentiated cortical cells. They have mitochondria, lipid droplets, SER, free ribosomes (some are arranged in polyribosomes) and Golgi complex (fig 4.11, 4.12).

4.2.2.2.3 Morphogenetic Features

This section will give details about microtubules (MT), microfilaments (MF), cell surface shape and junctions.

The cell junctions are well preserved in both cortical and chromaffin cells. Gap junctions, desmosomes as well as intercellular spaces are seen between like and unlike cells (fig 4.12). Both cortical and chromaffin cells tend to have more and wider intercellular spaces when abutting blood vessels.

Microtubules (MT) and microfilaments (MF) are present within the cytoplasm of both cortical and chromaffin cells of the adrenal gland (fig 4.12). Both MT and MF are seen more frequently

among light cortical cells in all studied stages (fig 4.11), but this may be due to the relative emptiness of their cytoplasm, making MF and MT more easy to observe.

Over the period studied (15-19 day chick embryos), intercellular spaces, junctions, MT and MF distribution showed no obvious differences.

The cell surface in all adrenal gland cells is smooth. Almost all cortical and chromaffin cells have a polygonal shape, though some chromaffin cells have an elongated shape. These elongated chromaffin cells do not show any signs of locomotory processes and have similar features to those of polygonal shape.

4.2.2.2.4 Cell Death

The TEM work confirms the presence of dying cells within adrenal gland tissues. Dying cells are present during the 17-19 day period in both chromaffin and cortical tissues (fig 4.13, 4.14). They are characterised by darkly stained cells with very darkly stained nuclei. They also have an irregular shape with some nuclei being very shrunken and folded (fig 4.14). The cells have a vacuolated cytoplasm and their cytoplasmic organelles have a bizarre appearance (fig 4.14). The dying cells generally appear to retain contacts with their neighbours in these embryonic stages (fig 4.13, 4.14). There is no signs of macrophages in the area of cell death.

4.2.2.3 Scanning Electron Microscopy (SEM)

The aim of the SEM work was to observe cell shape and surface structure. This work was mainly done on embryonic tissues (15 and 19 day).

No matter what stage or which fracturing method was used,

the chromaffin and cortical tissues appeared very compact with cell shape and cell boundaries not identifiable (fig 4.15). On the other hand, blood vessels and blood cells were well preserved (fig 4.16). The endothelial cells look flat and appear to be overlapping. The cells boundaries are easily noted and the cellular attachment between endothelial cells are provided with microvilli which reach over neighbouring cells (fig 4.16).

Fig. 4.1. Camera lucida drawing showing embryonic avian adrenal gland position within the urinogenital region.

A=aorta, LA=left adrenal gland, LG=left gonad, LK=left kidney, RA=right adrenal, RG=right gonad, RK=right kidney

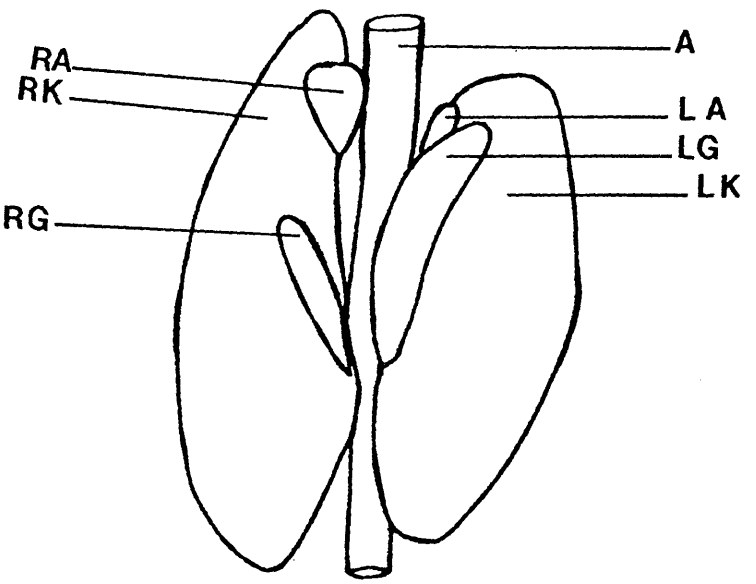


Fig.4.1

Fig. 4.2. Camera lucida drawing of a section of a 17 dce adrenal showing the distribution of chromaffin tissue (dark) among cortical tissue (clear). This section is from my own work. (Bar=133.3 μm)

dce=day chick embryo

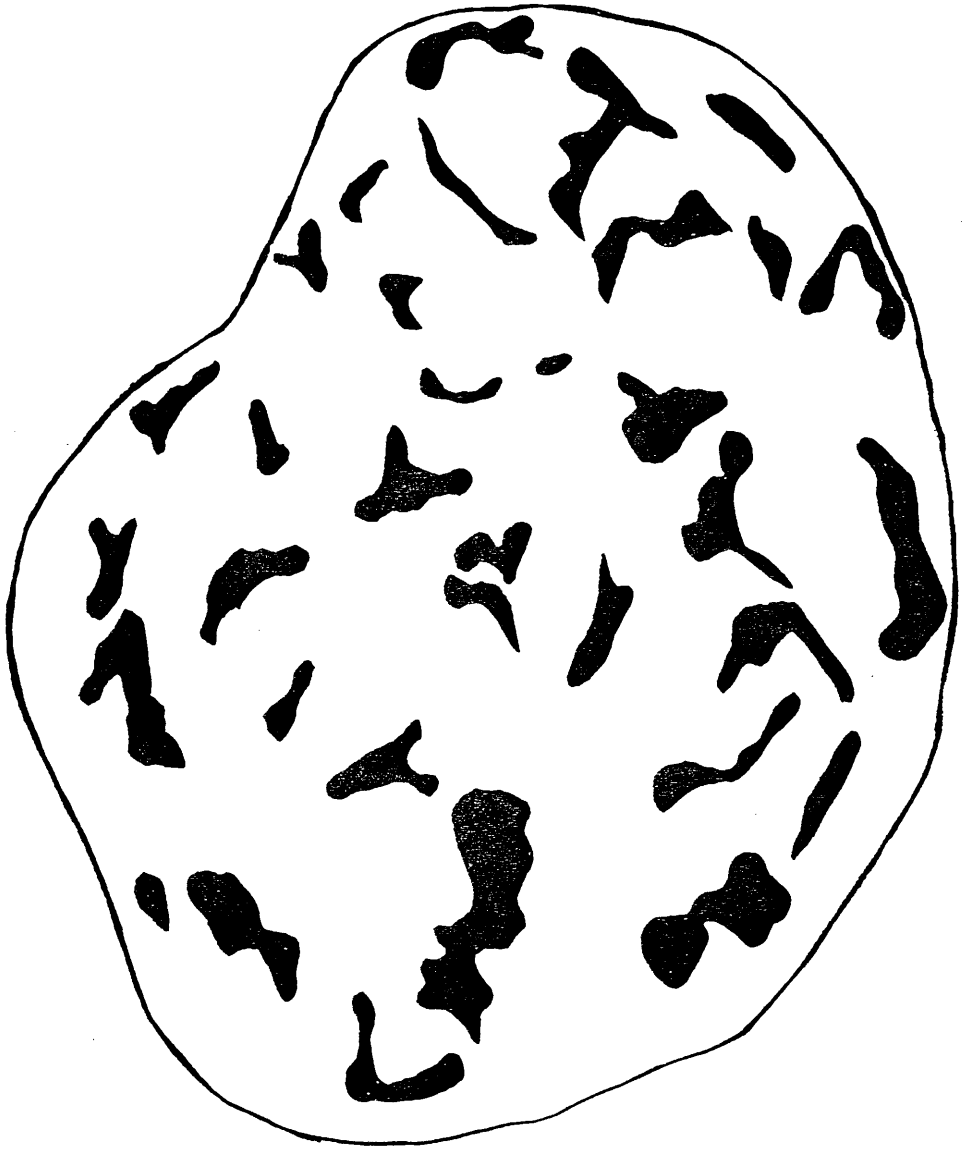


Fig. 4.2

Fig. 4.3. Transverse section through 19 dce adrenal showing chromaffin cells (arrows) around cortical cords (CC). (Ar+Asc). (Bar=25 μm).

Fig. 4.4. Transverse section through 15 dce adrenal showing some chromaffin cells (arrows) under the capsule of the gland and around cortical cords (CC). (Ar+Tb). (Bar=25 μm).

Fig. 4.5. Transverse section through 19 dce adrenal showing chromaffin cells (arrows)in association with the sympathetic ganglion cells (SG).It also shows chromaffin cells under the capsule (X). Nerve fibres are shown by arrow heads. (Ar+Tb). (Bar=50 μm).

Fig. 4.6. Transverse section through 10 dph adrenal showing two types of chromaffin cells small arrows indicate NA and large arrow indicates A. (Ar+Asc). (Bar=25 μm).

Ar=araldite resin, Asc=ammoniacal silver carbonate, bv=blood vessel, CC=cortical cells, dce=day chick embryo, dph=day post-hatching, SG=sympathetic ganglion cells, X=capsule

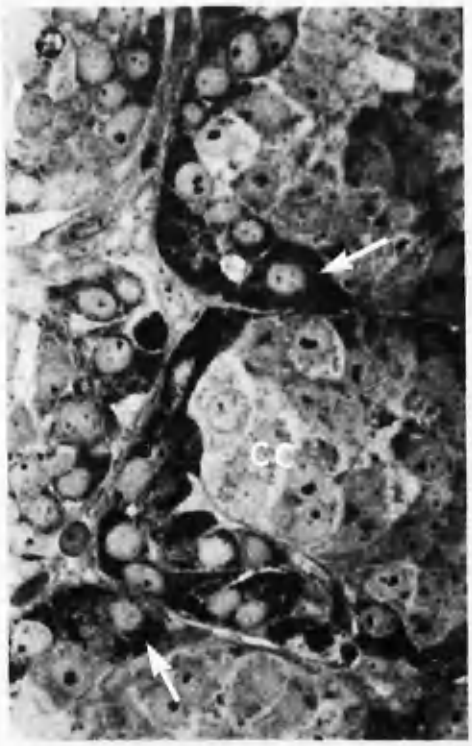


Fig.4.3

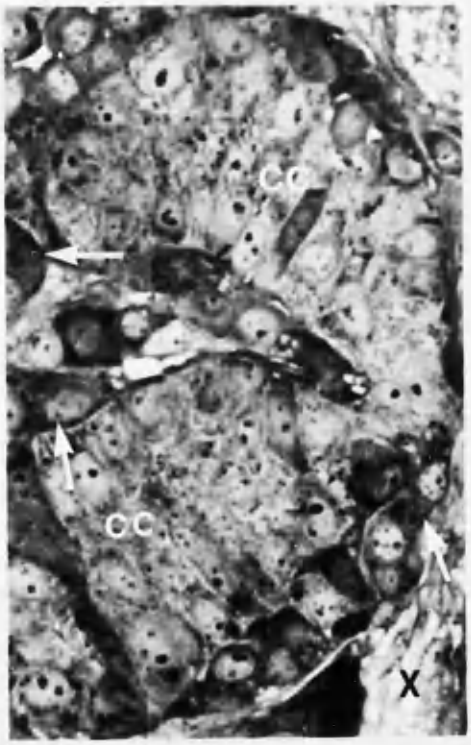


Fig.4.4

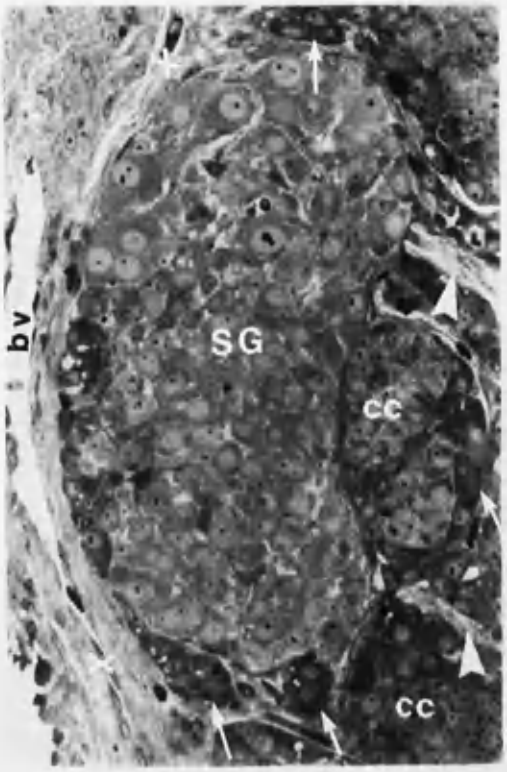


Fig.4.5

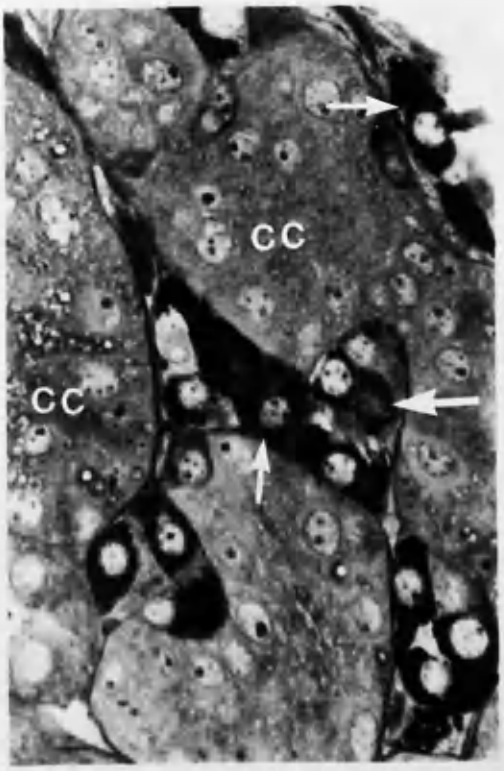


Fig.4.6

Fig. 4.7. Transverse section through 15 dce adrenal showing light (small arrows) and dark (large arrows) cortical cells. (Ar+Tb). (Bar=25 μm).

Fig. 4.8. Transverse section through 15 dce adrenal showing a group of sympathetic ganglion cells (SG) inside the gland. Light cortical cells are indicated by arrows and chromaffin cells by arrow heads. (Ar+Tb). (Bar=25 μm).

Fig. 4.9. Transverse section through 19 dce adrenal showing dying cells (arrows). (Ar+Tb). (Bar=25 μm).

Ar=araldite resin, cc=cortical cells, dce=day chick embryo, SG=sympathetic ganglion cells, Tb=toluidine blue

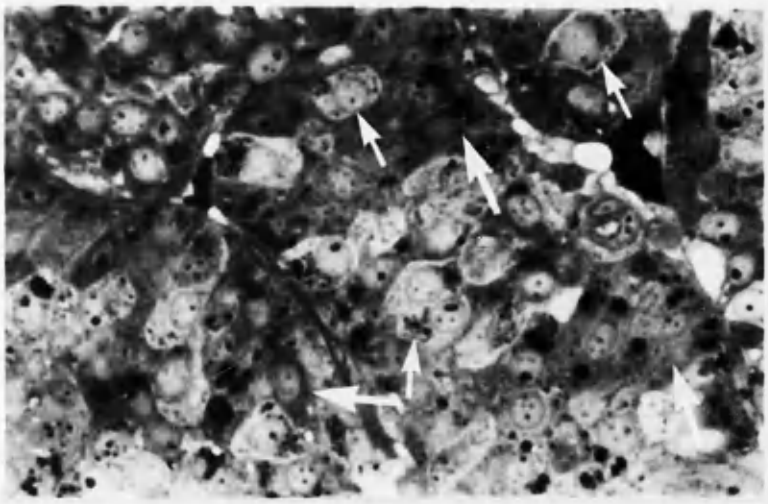


Fig.4.7

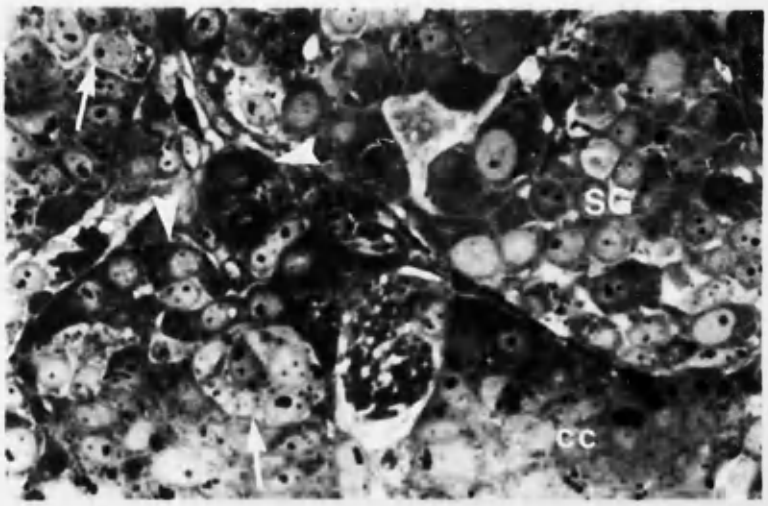


Fig.4.8

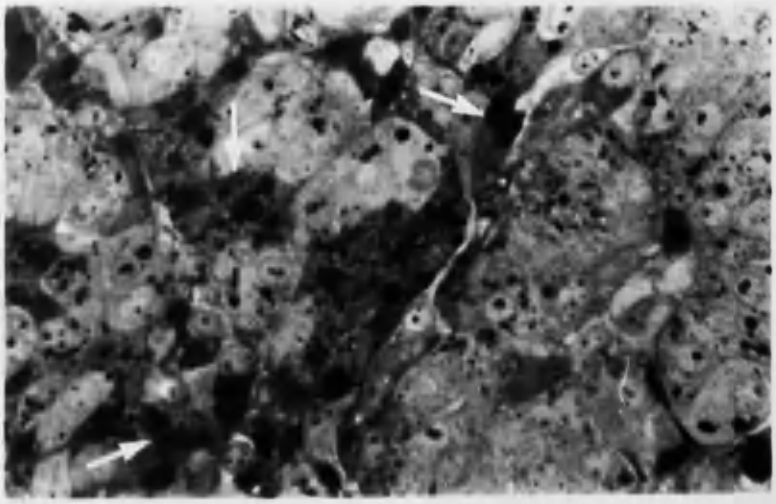


Fig.4.9

Fig. 4.10. Electron micrograph of 19 dce adrenal showing chromaffin cells (M), cortical cells (C) and blood vessel (bv). Chromaffin cells (M) show variable sizes of chromaffin granules (large arrows) and compact mitochondria (small arrows). Cortical cells show large mitochondria (m) and lipid droplets (lp). (Bar=3.13 μm).

Fig. 4.11a. Electron micrograph of 17 dce adrenal showing light (LC) and dark (DC) cortical cells. (Bar=3.13 μm).

Fig. 4.11b. An enlargement of the box in Figure 4.11a. of a light cortical cell (LC) showing microfilaments (small arrows) and microtubules (large arrows). (Bar=0.81 μm).

bv=blood vessel, C=cortical cells, DC=dark cortical cells, dce=day chick embryo, LC=light cortical cell, lp=lipid droplets, M=chromaffin cells, m=mitochondria, n=nucleus

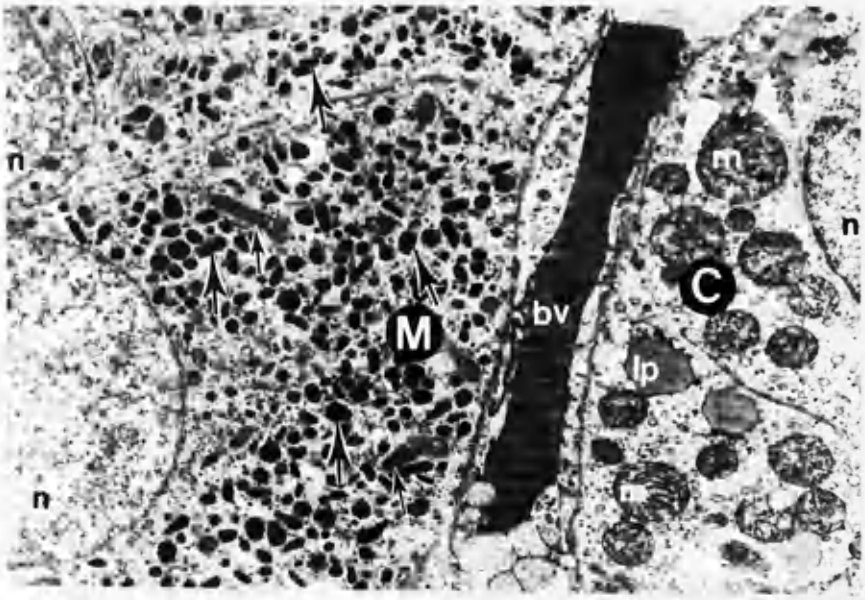


Fig. 4.10

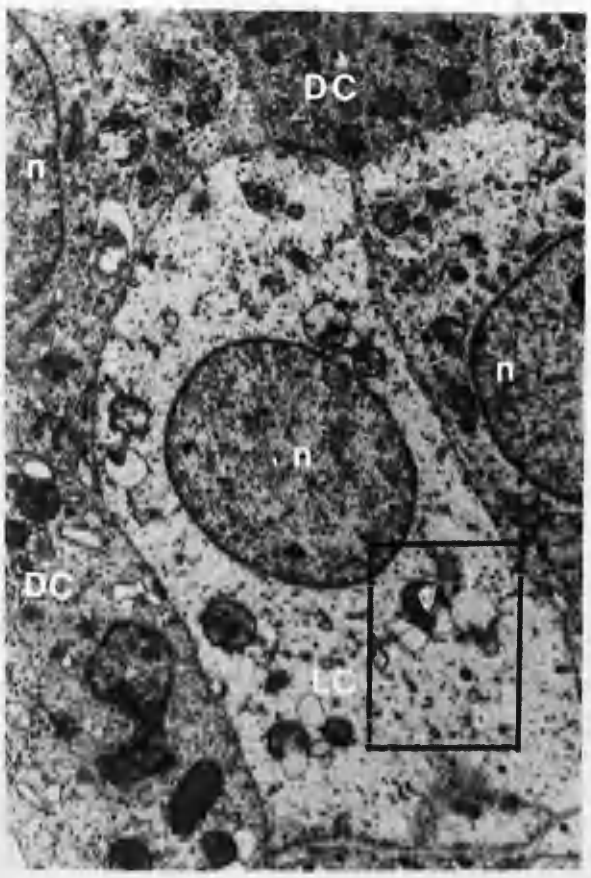


Fig. 4.11a

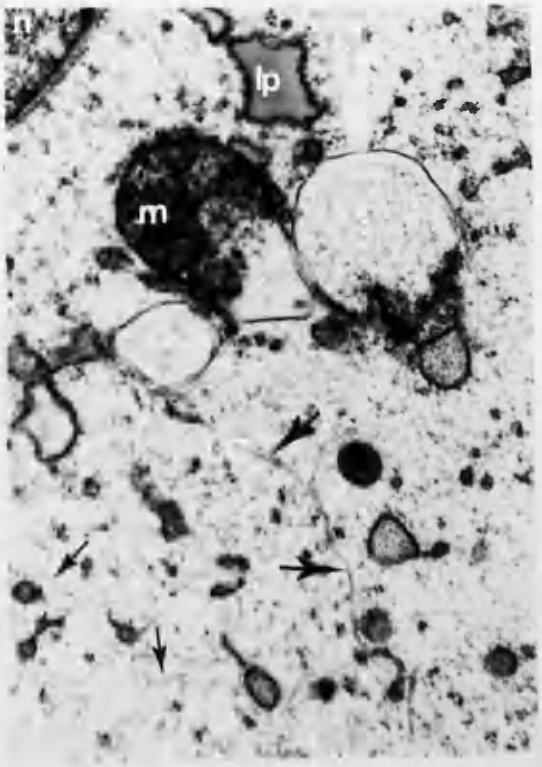


Fig. 4.11b

Fig. 4.12. Electron micrograph of 19 dce adrenal showing cell junctions between cortical cells. Desmosomes and desmosome-like junctions are indicated by large arrows; probable gap junctions by small arrows. (Bar=0.81 μm).

Fig. 4.13. Electron micrograph of 19 dce showing a dying chromaffin cell (dc). (Bar=3.13 μm).

dc=dying chromaffin cell, dce=day chick embryo, m=mitochondria, n=nucleus

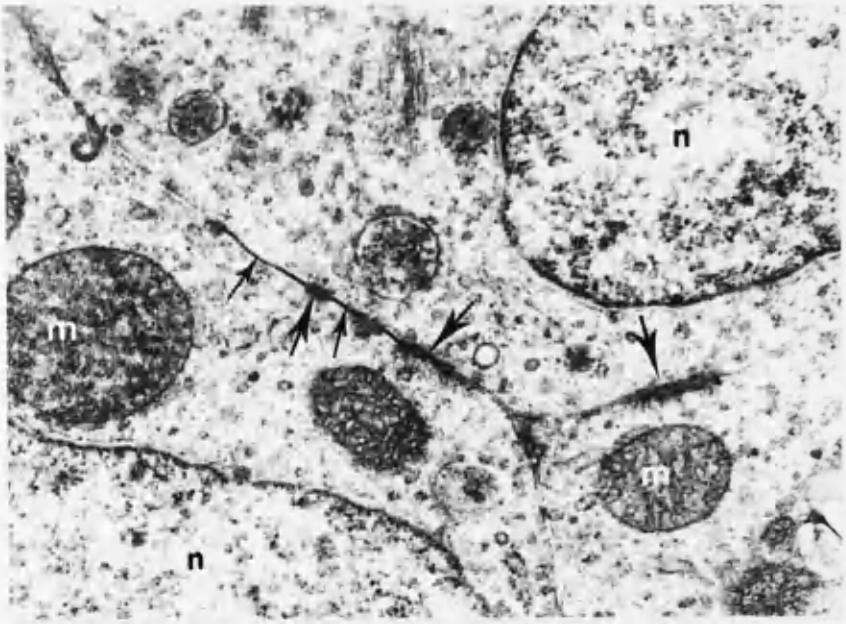


Fig.4.12

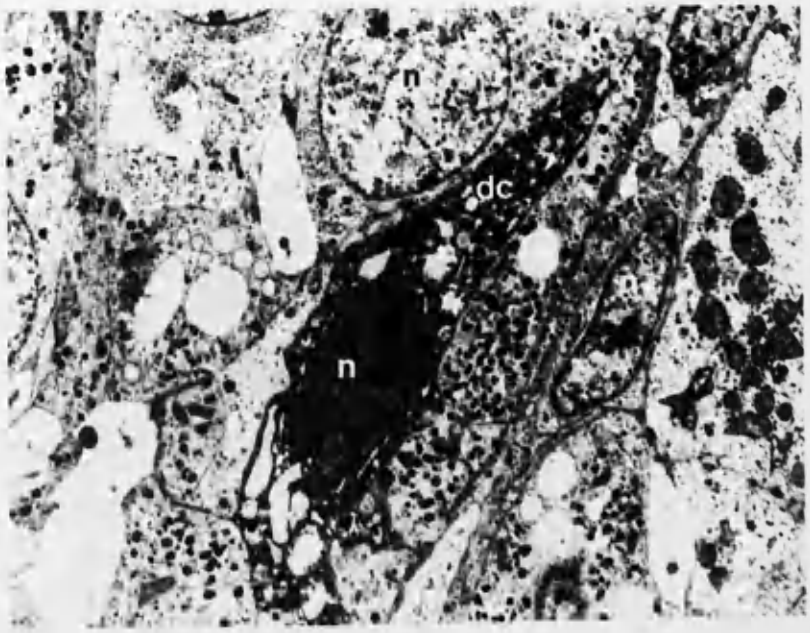


Fig.4.13

Fig. 4.14. Electron micrograph of 19 dce showing dying cortical cells (dc). Arrow heads indicate mitochondria of the dying cortical cells (dc). (Bar=3.13 μm).

Fig. 4.15. Scanning electron micrograph of 15 dce showing endothelial cells large arrows with filopodia small arrows of a blood vessel (bv). (Bar=19.6 μm).

bv=blood vessel, CC=cortical cells, dc=dying cortical cells, dce=day chick embryo, n=nucleus

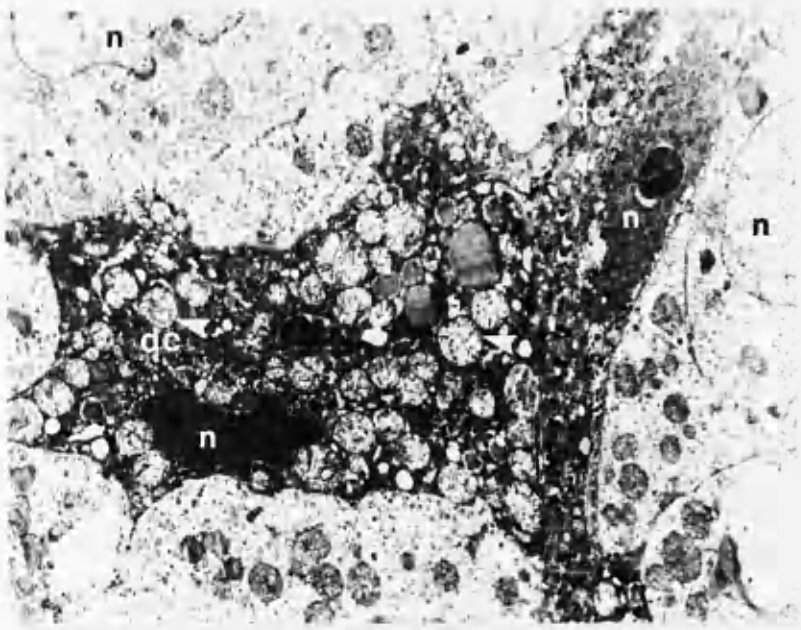


Fig.4.14

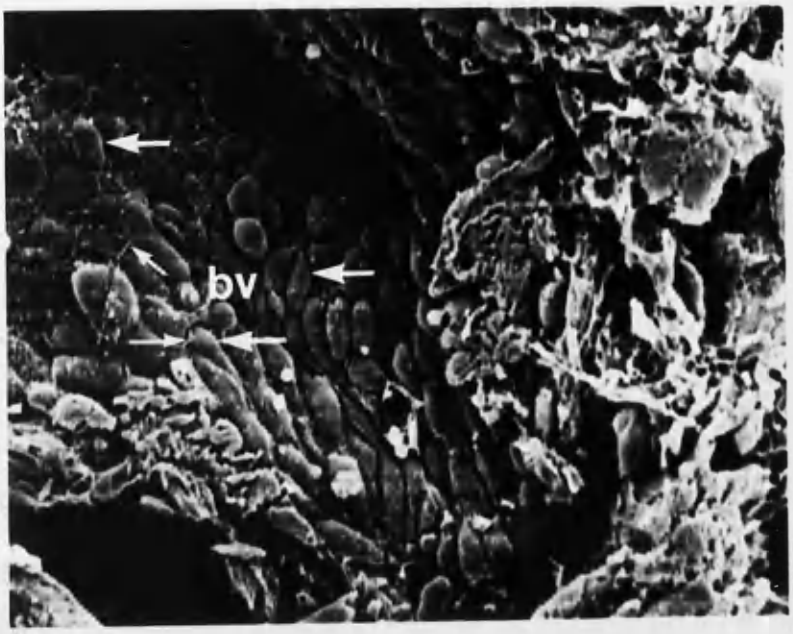


Fig.4.15

4.3 Discussion

This work on chick adrenal gland development is concerned with later stages of development (15-19 days of incubation and 10 days post-hatching). This section will summarise and discuss the main features found in this study. For convenience these will be divided into subsections: chromaffin tissue organisation, cortical tissue organisation, ultrastructural features and cell death.

In the chick, the coming together of chromaffin and cortical components occurs much earlier than in the mouse. The chick adrenal gland was less compact during early stages of this study, but became more compact near hatching.

The general structure of the adrenal gland showed only minor differences in overall organisation between embryonic and post-hatching stages.

4.3.1 Chromaffin Tissue

Chromaffin cells were present as individuals or in groups. Single cells were abundant during early stages of this study, and less abundant in late embryonic stages and post-hatching. Chromaffin cells arranged in groups were abundant in later stages of this study, though single cells were still present, even in post-hatching adrenals. Hall & Hughes (1970) found that chromaffin cells occur as single cells at about the 10th day of incubation, but that the majority of these cells were arranged in groups by the 18th day of incubation. They did not comment on the presence of single chromaffin cells in late embryonic stages.

The arrangement of chromaffin cells into groups may be due to (a) cell division with a lack of separation of products, or (b) aggregation of individual cells to form a group or (c) cell

differentiation from sympathetic ganglion cells. The last two proposals may be behind chromaffin cell grouping.

It might be possible to distinguish whether aggregation of small groups and single cells is occurring by counting the number of groups at different stages of embryonic life.

Chromaffin cells show some association with sympathetic ganglionic cells. This association is very obvious from the 16th day of incubation onward. These sympathetic cells are present inside and outside the gland. Since no more cells migrate into the gland and mitotic activity was not abundant within chromaffin tissue in these late embryonic stages, sympathetic ganglion cells may contribute to the increased number of chromaffin cells in later stages of development, by means of sympathetic cells differentiating into chromaffin cells. Unsicker (1973a) discussed the presence of sympathetic ganglion cells in adult domestic fowl. These cells occur as single or as scattered groups within the gland. A large number of these ganglion cells are situated near the surface of the gland and surrounded by their own connective tissue layer. Unsicker (1973a) showed that there are two types of ganglionic cells, large and small cells and that small ganglionic cells are seen among the chromaffin cells. He showed that these small ganglionic cells are in a transitional stage, and will finally differentiate into chromaffin cells.

4.3.2 Cortical Tissue

The cortical tissue is arranged in cords in both the embryonic and post-hatching stages studied. The cortical cords are surrounded by thin connective tissue, separating them from the surrounding tissues, such as other cortical cords, blood

vessels, sympathetic ganglion cells and chromaffin tissue. Hall & Hughes (1970) found that cortical cords are distributed evenly throughout the gland.

On the basis of staining reaction and overall arrangements, embryonic and post-hatching adrenals showed no obvious zonation. The cortical cells of the periphery and centre of the adrenal looked the same and showed no difference with respect to their ultrastructure. Zonation has been claimed by Sivaram (1965) on the chick embryo; Kjaerheim & Kondics (1966) and Aire (1980) on young and adult fowl; Haack *et al* (1972) on adult duck and Unsicker (1973c) on several adult avian species. They based their claims on the differences of ultrastructural features in cortical tissues.

I observed two cortical cell types, light and dark cells. The light cells were seen on the 15th day of incubation and in all later embryonic stages studied. I could not see any light cells in post-hatching stages. The light cells were characterised by a clear cytoplasm, lack of vacuoles and paucity of cytoplasmic elements. They had occasional lipid droplets and some mitochondria and dilated rough endoplasmic reticulum. Hall & Hughes (1970) also found two types of cortical cells, dark and light cells, at about the 17th day of incubation. They claimed that the light cells represent an emptying phase in the secretory cycle. My observations are in agreement with those of Hall & Hughes (1970).

4.3.3 Ultrastructural Features

Cortical cells of embryonic stages studied possessed lipid droplets indicating that cells were already differentiated. On the other hand, embryonic chromaffin cells had both moderately

dense and dense granules (A and NA granules) indicating that chromaffin cells were still undifferentiated. There have been several reports describing avian adrenal gland tissue differentiation. Sivaram (1965) showed that cortical cells become functioned on the 10th day of incubation and chromaffin cells on about the 11th day when they start secreting NA. He showed that A and NA granules are present in the same embryonic chromaffin cells in all stages studied and that chromaffin cells become differentiated after hatching. Mastrolia & Manelli (1969) claimed that chromaffin cells are differentiated into A and NA cell types by the 15th day of incubation. Hall (1970) and Hall & Hughes (1970) showed that chromaffin cells become differentiated after 17 days of incubation. My observations on chromaffin cell differentiation agreed with those of Sivaram (1965).

The cell surface is smooth in all embryonic tissues and I saw no sign of the sort of elongated cell processes expected in actively moving cells. The distribution of gap junctions and desmosomes showed no obvious differences over the period studied.

The possibility of active aggregative cell movements in both mouse and chick adrenals will be discussed in chapter 8.

4.3.4 Cell Death

One aspect of chick adrenal gland development which has not been described before, except in the work of Sivaram (1965), is the occurrence of cell death. Sivaram (1965) stated that cell death occurs in the centre of young and adult adrenal glands. His view was that peripheral cortical cells migrate inwards to the gland centre, to replace dying cells.

In my study, cell death occurred throughout the gland as

individuals and in groups, and involved both cortical and chromaffin cells. There was no sign of macrophages near the dying cells and I believe that neighbouring cells may do the work of the macrophages in clearing up the debris of these dying cells.

The occurrence of cell death in adrenal development, both in mouse and chick will be generally discussed in chapter 8.

CHAPTER FIVE

Distribution of Fibronectin in the Developing Adrenal Gland

5.1 Introduction

Fibronectin is a high molecular weight, fibre-forming glycoprotein. Fibronectin is the second most abundant glycoprotein in extracellular matrix, collagen being the most abundant. Fibronectin was first discovered around 1974 in several laboratories (Trinkaus, 1984). It occurs in two different forms, a cellular and a plasma fibronectin (Yamada & Olden, 1978 and Yamada,1983). Cellular fibronectin is present on cell surfaces as insoluble fibrils and also in extracellular matrix and basement membranes. Plasma fibronectin is present as a soluble protein circulating in the blood. The blood form of fibronectin had long previously been known as "cold insoluble globulin" (CIG). Both types of fibronectin have a similar composition and structure but are not identical (For reviews, see Alberts *et al*, 1983; Icardo & Manasek, 1983; Yamada, 1983; Harrisson *et al*, 1984; Hynes, 1986; Sternberg & Kimber, 1986 and Darribere *et al*, 1985).

Fibronectin is not evenly distributed in adult tissues. In a survey of fibronectin distribution in adult human tissues, using indirect immunofluorescence, Stenman & Vaheri (1978) found fibronectin in basement membranes that underlie epithelial cells of some organs such as gastrointestinal tract, respiratory tract, urinogenital tract and some glands such as thyroid, and mammary gland. It was also present around smooth muscle cells and striated muscle fibres. It was found generally in loose

connective tissue, such as capsules of glands, blood vessels walls, stroma of lymph nodes and spleen and the connective tissue of nerve bundles. In solid glands such as parathyroid and liver, fibronectin was not found between the glandular cells, but only around blood vessels and other connective tissue areas. Fibronectin was present in the skin both in dermis (connective tissue) and epidermis (basement membrane). Stenman & Vaheri (1978) did not study fibronectin distribution in the adrenal gland. To my knowledge, no previous work had been done on the presence of fibronectin in the adrenal gland neither in adults nor in embryos.

Fibronectin has been detected at very early stages in different embryos. In mammals, Wartiovaara *et al* (1979) showed that fibronectin is not detected in early preimplantation stages but is first found between cells of the inner cell mass of the late blastocyst stage. They argued that the onset of fibronectin expression in the late blastocyst stage coincides with the appearance of the primitive endoderm.

In chicks, Critchley *et al* (1979) and Harrisson *et al* (1984) both showed that fibronectin is present during gastrulation. It is present between the ectoderm and endoderm of both area pellucida and area opaca, including the ventral surface of the epiblast (upper layer), anterior to Henson's node and lateral to the primitive streak. In later stages of development, Mayer *et al* (1981) reported the presence of fibronectin between the cell layers of the area vasculosa, around the notochord, and next to the neural tube before the migration of the mesenchymal cells from the somites into these areas.

In amphibians, Boucaut & Darribere (1983) and Lee *et al* (1984) showed that fibronectin is present at the start of

gastrulation especially in the roof of the blastocoel. Boucaut & Darribere (1983) claimed that fibronectin fibrils are present in the blastocoel roof prior to the migration of the mesodermal cells; that is, fibronectin fibrils develop in the early blastula stage. Lee *et al* (1984) argued that fibronectin is synthesised at a low rate from maternally derived mRNA during oogenesis but kept within the egg, and secretion of fibronectin increases rapidly during late blastula and early gastrula stages.

Fibronectin is found to promote cell -cell and cell- matrix interactions, motility of certain cells and embryonic differentiation (Yamada & Olden, 1978; Critchely *et al*, 1979; Icardo & Manasek, 1983; Thiery *et al*, 1985 and Sorrell, 1988).

Fibronectin rich pathways guide and promote the migration of many kinds of cells during embryonic development and also position cells in their definitive location (Mayer *et al*, 1981; Alberts *et al*, 1983; Hynes, 1986; Sternberg & Kimber, 1986 and Darribere *et al*, 1988).

The functions proposed for cellular fibronectin relate to cell migration and differentiation during development. Cellular fibronectin is found to restore adhesiveness and normal morphology to transformed cells (Yamada & Olden, 1978). The functions proposed for plasma fibronectin are related to the migration of blood cells within the extravascular spaces and the removal of collagenous and other debris from blood after injuries via sessile macrophages of the reticuloendothelial system (Yamada & Olden, 1978 and Sorrell, 1988). Both types of fibronectin display adhesivity. Much of the evidence on the functional aspects of fibronectin is gathered from *in vitro* work. When fibronectin is added to cultured cells, the cells adhere rapidly

to the substrate and flatten out (Hynes, 1986). When cells adhere *in vitro* in the presence of fibronectin, internally, the cells have well organised actin filament bundles associated with adhesion points (Alberts *et al*, 1983).

Fibronectin may be able to direct the movement of cells. Evercoorn *et al* (1982) suggested that fibronectin may be a chemo-attractant agent for Schwann cells. They precoated filters with fibronectin which they then placed in Boyden chambers. The chambers contained suspended Schwann cells with medium placed on top and attractant solution on the bottom. They found that cells migrate at a maximum speed toward a positive gradient of fibronectin whereas less motility was seen when an equal concentration of fibronectin was present on both sides of the filter. This suggests that fibronectin gradients could exist in embryos to direct the movement of cells. Though Evercoorn *et al* (1982) discussed their observations in terms of a soluble chemo-attractant gradient, it seems more likely that their results are due to an adhesive gradient of fibronectin. Adhesive gradients of this kind have long been postulated and demonstrated *in vitro*. A directional response to an adhesive gradient is normally termed a haptotaxis (Trinkaus, 1984).

The cell types found to make fibronectin in culture are fibroblasts, myoblasts, endothelial cells and amniotic cells (Critchley *et al*, 1979; Jaffe & Mosher, 1978 and Trinkaus, 1984). Critchley *et al* (1979) showed that when adult epithelial cells were cultured, they secreted fibronectin into the growth medium. *In vitro*, fibronectin is also synthesised and organised into fibres by the ectoderm, endoderm, somites, notochord, neural tube (Newgreen & Thiery, 1980).

The amount of fibronectin present within a tissue depends on the state of that tissue. Yamada & Olden (1978) showed that fibronectin is low when cells are in mitotis and also when embryonic cells start to differentiate. But its level is high when embryonic cells are migrating and also during wound healing (Hynes, 1986).

Leptin (1986) stated that cells can carry two separate fibronectin receptors with different specifities, or may have only one receptor. These receptors differ in their affinity and specifity to fibronectin (Leptin, 1986 and Darribere *et al*, 1988). The receptors can be either strong or weak depending on the cells and their environment. The receptors organise fibronectin into fibres and bind to it, hence forming a "foot" which enables a cell to move forward (Bretscher, 1987).

One type of embryonic cell that has been looked at intensively with respect to fibronectin functions is the neural crest. Neural crest cells use the extracellular matrix, which is rich in fibronectin, as their guide during migration. Fibronectin also plays a role in positioning these cells in their final positions (Newgreen & Thiery, 1980; Harrison *et al*, 1984; Thiery *et al*, 1985; Hynes, 1986 and Leptin, 1986). Fibronectin is laid down prior to neural crest cell migration by the surrounding tissue such as the mesenchymal cells (Trinkaus, 1984; Newgreen & Thiery, 1980 and Thiery *et al*, 1985). Neural crest cells are not able to synthesise fibronectin nor able to organise fibronectin on their surfaces, but they do have fibronectin receptors which are uniformly distributed on their surfaces (Trinkaus, 1984 and Thiery *et al*, 1985).

Newgreen & Thiery (1980) showed that *in vivo*, the trunk neural crest cells of early chick embryos migrate in

fibronectin-rich extracellular matrix, but in later stages, rate of migration and the number of migrating cells is related to the amount of fibronectin present.

The adrenal gland consists of two different tissues, the cortical and chromaffin. The chromaffin tissue has a neuro-ectodermal origin. It is the neural crest cells of the trunk region which give rise to the chromaffin tissue of the adrenal gland.

The aim of this study was to find the distribution of fibronectin in the adrenal gland of mouse and chick embryos at different stages in order to discover whether fibronectin might be in any way responsible for the migration of the chromaffin cell within the developing adrenal gland. I have found no previous study of fibronectin distribution in the adult or embryo of mouse or chick adrenal glands.

5.2 Results

Chick and mouse adrenals were stained for indirect immunofluorescence with anti-fibronectin antibody as detailed in chapter 2 (section 2.4) . Two different fixation procedures were tried. No positive results were obtained with paraformaldehyde fixation, though it is not clear why this should be so. Good results were obtained with ethanol fixation.

All studied tissues showed a positive reaction to the fibronectin antibody but differed in their staining intensity. Control sections showed no fluorescence.

5.2.1 Mouse Adrenals

The study of fibronectin distribution was carried out on three stages (14, 16 and 18 day embryos). Fibronectin was seen distributed in the capsule area, blood vessels and around clumps of cells within the gland (fig 5.1, 5.2). Fibronectin distribution is seen more in the interior parts of the gland than in the capsule area.

Fibronectin was seen as a bright green fluorescent sheet around the cortical cell clumps both at the periphery and the centre (fig 5.1, 5.2). It was also present within a few cortical clumps as thin weakly-stained strands but this was uncommon. Fibronectin was present around chromaffin cell patches as described for cortical tissue clumps and also around some isolated chromaffin cells (fig 5.1, 5.2) but not within chromaffin cell clumps. The lumen of blood vessels and sinusoids fluoresced strongly presumably due to the presence of soluble fibronectin. Because of the strong fluorescence of blood vessels contents, it was difficult to distinguish the presence of fibronectin in blood vessels walls.

Fibronectin staining intensity was greater in 18 day mouse embryos than in earlier stages.

5.2.2 Chick Adrenals

The study of fibronectin distribution was carried out on three stages (15, 17 and 19 day embryos). All three stages showed equal brightness of fluorescence. Fibronectin was seen distributed in the capsule area of the gland, blood vessels and cell groups within the chick adrenal gland (fig 5.3, 5.4).

Fibronectin was present around the cortical cords as a sheet

and also seen as thin strands of weak fluorescence within the cortical cords (fig 5.3, 5.4). Fibronectin was also seen around chromaffin cell groups as sheets and as strands of fluorescence, (fig 5.3, 5.4), but not within chromaffin groups. Fibronectin was present around sympathetic ganglion cells and nerve fibres which are present within the gland (fig 5.3, 5.4). These nerve fibres and sympathetic ganglion cells are not so easy to distinguish in mouse adrenal gland. It was also present within blood vessels as a thick band of bright fluorescence (fig 5.3).

Fig. 5.1a. Immunofluorescence micrograph of 18 dme showing fibronectin (arrow heads) distribution around the capsule (X), within cortical (c) and medullary tissue (m).

Fig. 5.1b. Control section of a similar area to Figure 5.1a.

Fig. 5.2a. Immunofluorescence micrograph of 16 dme showing fibronectin (arrow heads) in the central part of the gland, around chromaffin (m) and cortical (c) and within a blood vessel (bv).

Fig. 5.2b. Control section of a similar area to Figure 5.2a.

Bar=20.5 μm for all micrographs above.

bv=blood vessel, c=cortical tissue, dme=day mouse embryo, m=chromaffin tissue, X=gland's capsule

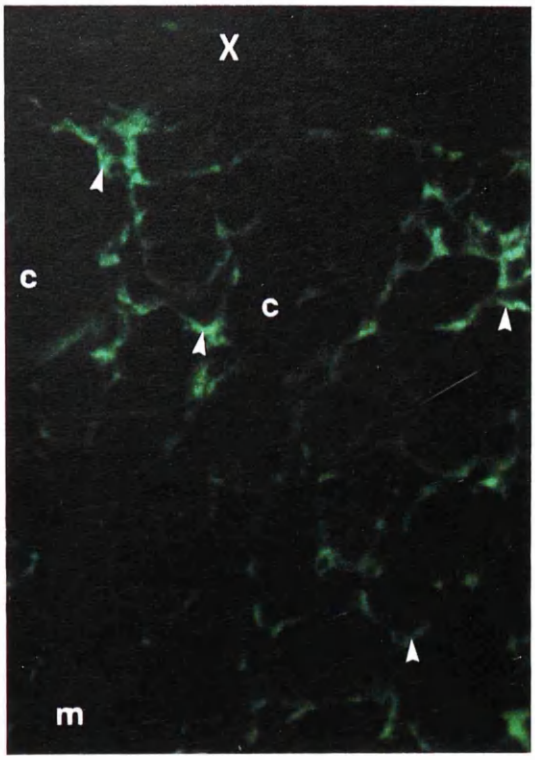


Fig.5.1a

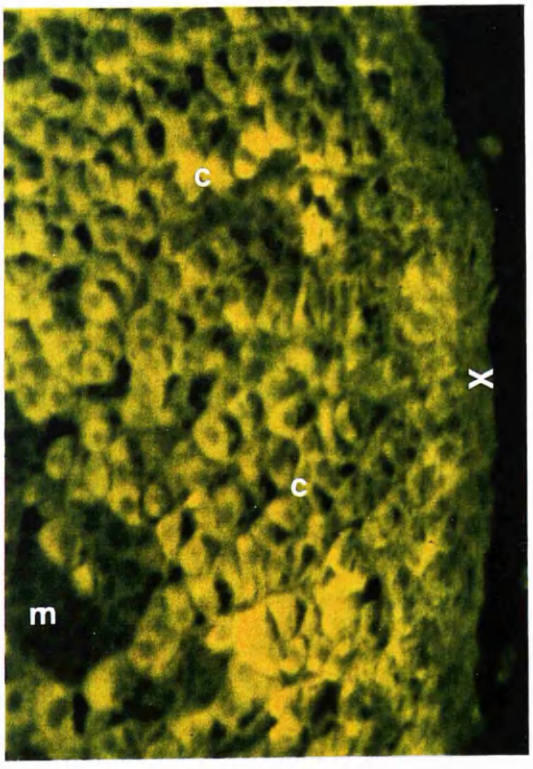


Fig.5.1b

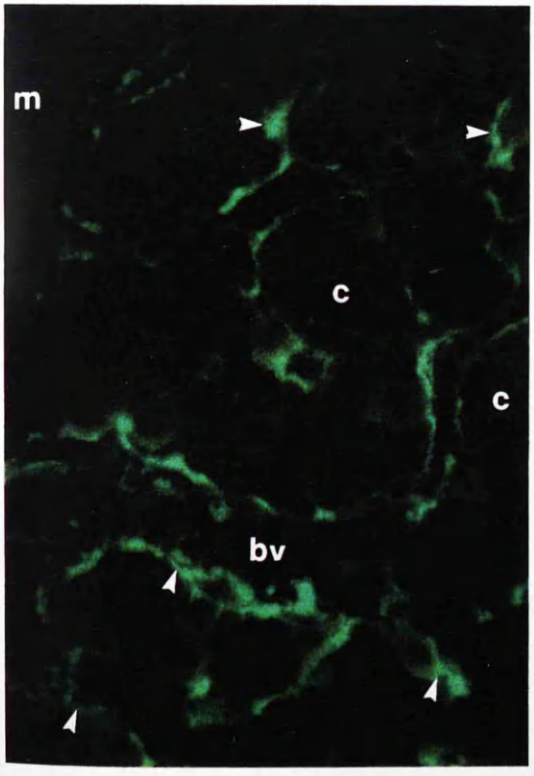


Fig.5.2a

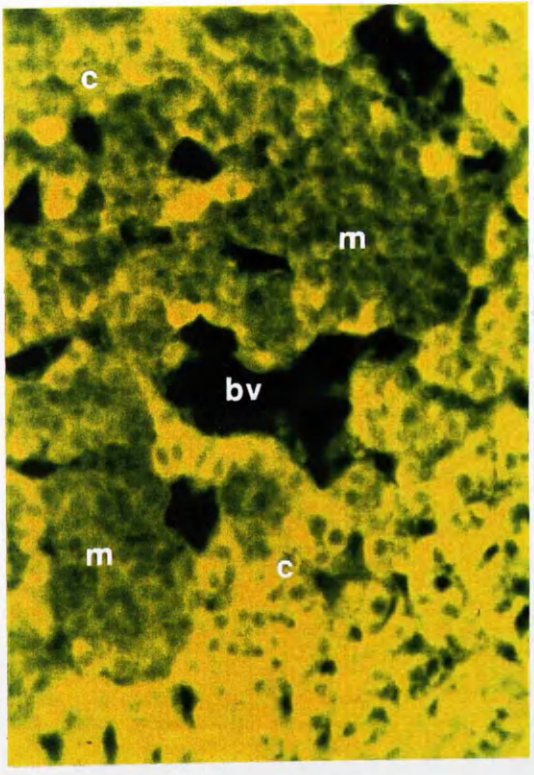


Fig.5.2b

Fig. 5.3a. Immunofluorescence micrograph of 19 dce showing fibronectin (arrow heads) between cortical cords (c), chromaffin group (m) and in blood vessels (bv).

Fig. 5.3b. Control section of a similar area to Figure 5.3a.

Fig. 5.4a. Immunofluorescence micrograph of 15 dce showing fibronectin (arrow heads) present among nerve fibres and cortical tissue (c).

Fig. 5.4b. Control section of a similar area to Figure 5.4a.

Bar=20.5 μm for all micrographs above.

bv=blood vessel, c=cortical tissue, dce=day chick embryo, m=chromaffin tissue, X=gland's capsule

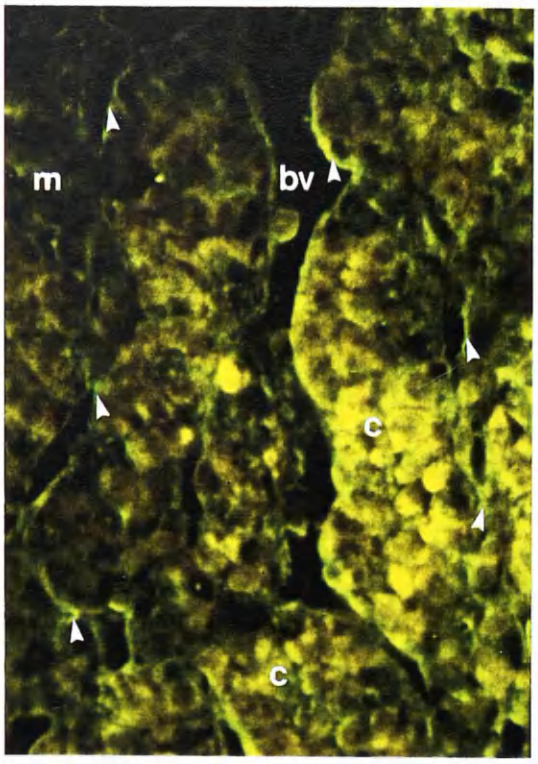


Fig.5.3a

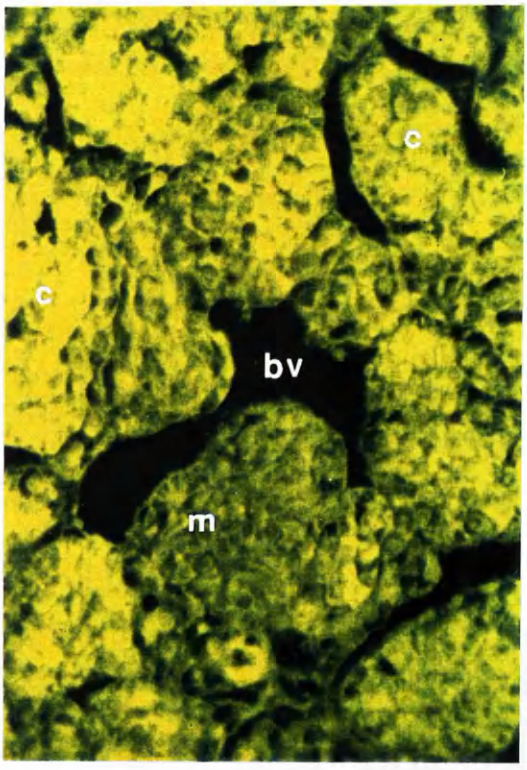


Fig.5.3b

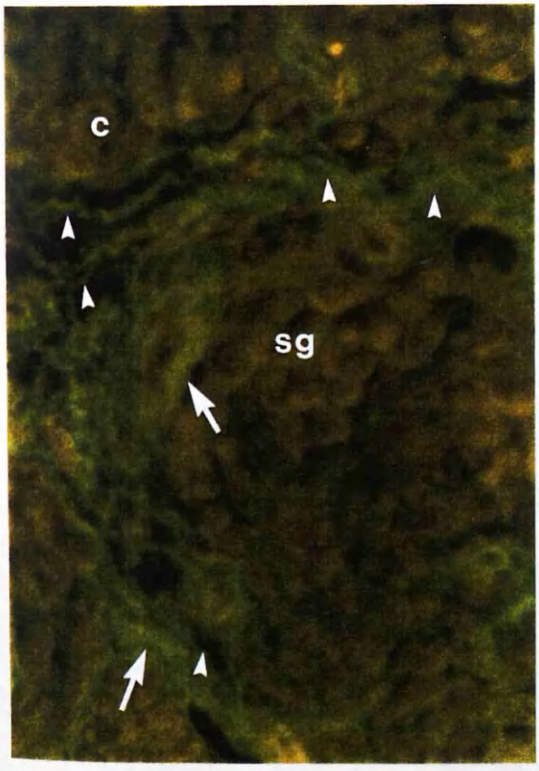


Fig.5.4a

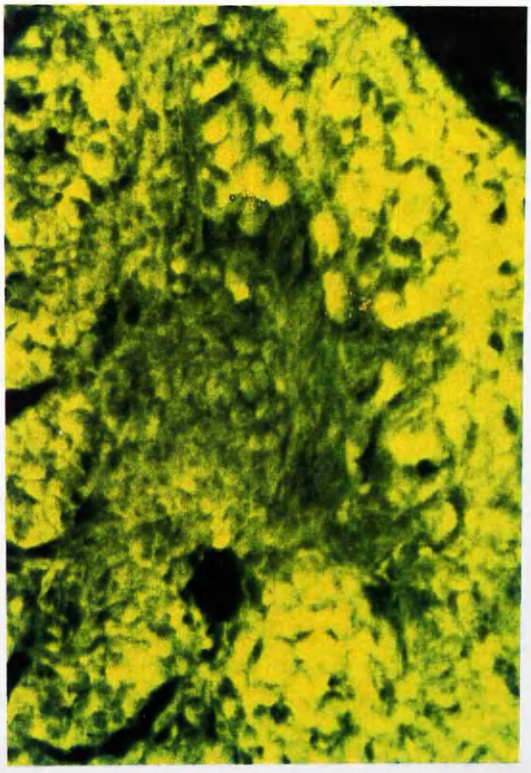


Fig.5.4b

5.3 Discussion

Although the embryonic and adult distribution of fibronectin has been studied, using indirect immunofluorescence, in several organ systems, no previous work seems to have been done on the adrenal gland either in embryonic or adult stages. Such a study is worth while because of the structure and development of the adrenal gland.

The present study was carried out on mouse embryo (14, 16 and 18 day) and chick embryo (15, 17 and 19 day) adrenal glands using indirect immunofluorescence to detect the localisation of fibronectin.

Fibronectin was detected in the capsule of the gland, between groups of both chromaffin and cortical cells and in blood vessels. Fibronectin was found between individual cells of cortical clumps, but to a much lesser extent between cells of chromaffin clumps. There may have been an increase in the amount of fibronectin in the gland in the 18 day mouse embryo, but otherwise there was no evidence in this study for a gradient of fibronectin, or any quantitative differences that might have been significant in gland morphogenesis.

The fibronectin in the adrenal gland is most probably produced by the mesenchymal cells which make part of the gland especially the capsule and also part of the cortex (Lever, 1955).

It is unlikely that fibronectin within the adrenal gland derives from chromaffin cells, since these cells are derived from the neural crest, and many studies have shown that neural crest cells migrate on fibronectin-rich substrates, but that they do not themselves secrete fibronectin (Thiery *et al*, 1985). My finding that fibronectin is found within cortical cell clumps, but not within chromaffin clumps supports this view.

Fibronectin guidance has been suggested for primordial germ cells of *Xenopus* embryos in the work of Heasman *et al* (1981), and also for mesodermal cell migration during urodele gastrulation (Nakatsuji, 1984). They suggested that fibronectin is secreted as fibrils and aligned along the migrating pathway and then used by the migrating cells as guidance cues to their final destinations.

It is possible that orientated fibrils of fibronectin could guide chromaffin cells to the centre of the mouse adrenal gland, but I have no evidence for this. As better methods come available for studying fibronectin orientation within solid tissues, it may be worth looking for this in the adrenal. It is highly unlikely, however, that such a guidance system could function in chick adrenal development, where chromaffin patches do not accumulate in the centre, but are dispersed throughout the gland.

A second way in which fibronectin could guide chromaffin cells to the centre of the mouse adrenal is as an adhesive gradient. Adhesive gradients have long been known to direct cell movements *in vitro* (haptotaxis- see Trinkaus, 1984), but evidence for them in embryos has remained sparse. However, a major hypothesis designed to explain mesenchymal condensation movements (Oster *et al*, 1983) relies on a combination of contact guidance and haptotaxis. There is good evidence for an adhesive gradient in the case of urodele pronephric duct elongation (Poole & Steinberg, 1982 and Zackson & Steinberg, 1986), and for fibronectin-based gradient in the case of heart endocardial cell migration(Linask & Lash, 1986). However, in my study, I have no evidence for a gradient of fibronectin in the adrenal, though it

is probable that the methods used are not fine enough to detect small differences in fibronectin concentration. It is, however, worth pointing out that the distribution of fibronectin in the mouse and chick adrenal gland is quite similar, yet chromaffin cells in chick do not converge on the middle of the gland. This comparative evidence argues against a fibronectin gradient.

Further study on the following aspects of fibronectin distribution in the adrenal could be useful:

a) Fibronectin distribution in adult adrenal glands; is it related at all to cell movement within the gland?

b) Fibronectin distribution in younger stages, at the time when chromaffin cells are just starting to migrate amongst the cortical cells.

CHAPTER SIX

Culturing of the Mouse Adrenal Gland Tissues

6.1 Introduction

Tissue culturing has been used to investigate cellular behaviour and functional aspects of very many types of cells and tissues in an artificial environment. This involves cultivating intact organs, single tissues or individual cells.

The development of tissue culturing techniques goes back to the beginning of this century. In 1910, Carrel & Burrows developed a technique which allows living tissues and organs to grow outside the body in an artificial environment. They used hollow glass slides as a culture environment, with plasma as growth medium. The plasma was extracted from the same animal as the tissues. Mammalian adrenal glands were one of the tissues which they used in their experiments. In 1929, Ssipowsky used the hanging drop method for culturing young rabbit adrenal gland (for review see Kahri, 1966). These early experiments were simply aimed at testing whether tissues could survive and grow in culture. The first experiments on adrenal gland function in culture were performed by Scheberg in 1955 .

There have been many more recent studies on adrenal tissues in culture. In most cases, these have involved the culturing of medullary or cortical tissues separately and their main aim has been to investigate functional aspects of the two tissues.

I will review these studies briefly in turn, commenting on how the tissues were isolated, what stage of tissues were used, what media were used and how the tissues behaved in culture. The

overwhelming majority of previous studies have been on postnatal adrenals. Previous culture work on pre-natal adrenals will be reviewed in section 6.1.3 of this chapter.

6.1.1 Chromaffin Tissue

Several methods of dissociation of chromaffin tissue have been employed. After cutting the tissue into small pieces, enzymes such as collagenase (Kilpatrick *et al*, 1980; Hesketh *et al*, 1981; Livett, 1984 on the bovine; Unsicker, 1981 on the guinea pig and Moore *et al*, 1982 on the rat) or trypsin (Moore *et al*, 1982 on the rat) or a mixture of these two along with DNAase (Notter *et al*, 1986 on the monkey) have been used. Among the growth media which have been used are 199 with serum (Unsicker, 1981 on the guinea pig and Moore *et al*, 1982 on the rat) and Dulbecco's modified Eagles medium (DMEM) with serum (Kilpatrick *et al*, 1980; Hesketh *et al*, 1981; Livett, 1984 on the bovine and Notter *et al*, 1986 on the monkey). The results of these studies showed that chromaffin tissues survived the enzymatic dissociation procedures and persisted in long term cultures as long as 3 weeks. According to Notter *et al* (1986) the use of the combined enzymes (collagenase and trypsin) gave a good yield of viable single cells and did not damage chromaffin cells as Malamed *et al* (1970) had claimed. When chromaffin cells were treated with nerve growth factor (NGF), the cells expressed significant sympathetic neuronal characteristics (Notter *et al*, 1986). Hesketh *et al* (1981) argued that when bovine chromaffin cells were cultured for a long period in the presence of nerve growth factor, they started to take on a neuronal appearance, with neurotic processes within 1-2 days of cultivation. Unsicker (1981) claimed that NGF and NGF-like growth factors may induce

neuronal transdifferentiation of adrenal chromaffin cells of newly born guinea pig. Notter *et al* (1986) pointed out that when NGF was added to monkey chromaffin cells in culture, cells took sympathetic neuronal like properties.

6.1.2 Cortical Tissue

Several methods of dissociation of adrenal cortical tissues have been used. Among these are the decapsulation of the adrenal gland and then microdissection of the cortex, along with the use of enzymes such as trypsin (Armato & Nussdorfer, 1972 on the rat).

The factors which are needed in the defined medium for maximum culture life span of adrenocortical cells are unknown (Simonian *et al*, 1987). Some of the growth media which have been used for culturing adrenal cortical tissues are Eagles minimum essential medium (MEM), Ham's F10 and F12 and 199, all with serum. The cultures have been kept as long as 2 weeks.

Armato & Nussdorfer (1972) using rat cortical cells, showed that MEM with fetal bovine serum gave the best results. They also showed that fibroblast-like cells and macrophages were the first to migrate out of the explants (3-5days of culturing). The cortical cells began to outgrow as columns on the 6th-8th day of cultivation. They also showed that large explants undergo necrosis within 3-5 days, but that smaller explants did not show any necrosis in that time.

Gospodarwicz *et al* (1977) showed that fibroblast growth factor (FGF) promotes the growth of bovine adrenal cortical cells. They claimed that it stimulates the mitogenic process in adrenal cortical while in culture. Fibroblast growth factor has

been shown to be required for adrenocortical cells proliferation throughout the finite life span in culture in serum-supplemented medium (Simonian *et al*, 1987). When epithelial cells of a rat adrenal gland were exposed to a medium which contains fetal calf serum (FCS), cells took on a fibroblastic appearance (Slavinski-Turley & Auersperg, 1978).

6.1.3 Culturing of Embryonic Adrenal Tissues

There have been several reports on the culturing of embryonic and newly born adrenal tissues. Among these are Kahri (1966) and Unsicker *et al* (1985) on fetal and new born rat adrenals and Kahri & Halinen (1974) on fetal human adrenals.

Kahri (1966) cultured small pieces of fetal and newly born rat adrenals in plastic dishes over a period of 2 months in a medium of 50% Melanick's A, 25% calf serum and 25% Parker's amino acids. Cells started to grow out of the explant within the first 24 hr of cultivation. These cells included fibroblasts, macrophages and epithelial cells. Degenerative signs were observed among the fibroblasts after the 3rd week of cultivation, whereas the epithelial cells showed a healthy appearance up to 2 months. Kahri & Halinen (1974) cultured small fragments of human fetal adrenal for as long as 20 days, in the same medium. They did not observe any outgrowth within the first 24 hr, but after 2 days cells started to grow out of the explants. Fibroblasts, macrophages and epithelial cells were all present, and mitosis was frequent among the outgrowing cells. Unsicker *et al* (1985) cultivated small pieces of embryonic rat adrenal gland (17, 19 and 21 day) on collagen-coated coverslips within plastic dishes, in medium 199 with or without fetal calf serum. The advantage of serum-free medium was that it inhibited fibroblast outgrowth but

the disadvantage was the loss of adhesiveness of the explants, hence it was more difficult to maintain the cultures. The aim of this work was to monitor medullary cell maturation. Cell types -A or NA- were identified using methods that allowed the type of chromaffin granule to be distinguished at light or electron microscopical level. For light microscopy, they used glyoxylic acid (De Le Torre & Surgeon 1976). They did not attempt positively to identify cortical cells. Cells started to grow within the first 24 hr.

6.1.4 Aims of the Present Work

The main aim of my work was to study the morphogenesis of mouse adrenal gland tissues *in vitro*. The work can be divided into two categories as follows:

1. Culturing of fragments of embryonic adrenals on glass or plastic substratum. The main aim was to investigate the interactions between chromaffin and cortical cells as they move out from the explant. I hoped that it would be possible to distinguish chromaffin and cortical cells in culture and that they might sort out from one another, forming a monolayer cortical-medullary arrangement, as in the sorting out experiments of Nicol and Garrod (1979) on liver parenchyma, limb bud mesenchyme, pigmented epithelium of the eye and corneal epithelium. Observations of this kind could help to determine the mechanism of the cortical-medullary morphogenetic process.
2. Culturing of halved and whole adrenals in suspension as a test of whether differential adhesion is part of the mechanism by which the adrenal tissues segregate from one

another.

6.2 Results

6.2.1 Cultures on Glass and Plastic

Adrenal gland cultures were made from the following stages: tissues of 15-18 day mouse embryos, 1-7 days old neonates, young adult and adult. The glands were cut into small pieces or into two halves or about 2/3 of the gland size. The tissues were cultured in small plastic petri dishes on glass coverslips, or on glass as hanging drops or in small plastic dishes all as described in section 2.5.3.1; 2.5.3.2; 2.5.3.3 and 2.5.3.4. The cultures were periodically observed with the aid of a phase contrast light microscope during the period of culture. The tissues were fixed after intervals of 1, up to 15 days. A short term culture (2 hr) was also carried out.

6.2.1.1 Small Pieces Culture on Glass and Plastic

Small pieces of embryonic adrenal glands were easy to maintain in culture whereas the adult tissues were not. It was hard to get the adult tissues to stick to the substratum. Although some did stick to the substratum they did not show any cell growth even when kept for as long as 4 days. After initial attempts, no further work was done using adult tissues. Hanging drop cultures of embryonic and neonatal adrenals were maintained for a maximum of 5 days, whereas coverslip cultures were kept for up to 15 days.

Cells began to move out from the edges of explants within 24 hr as single cells and started to spread and flatten out on the substratum. In the next few days more cells moved out of the

explant. Cells had a multipolar shape with long and short axon-like processes.

Unfortunately, it was very difficult to relate the appearance of the cells that emerged from the explants to the expectation of seeing cortical and chromaffin cells. Cells were observed by phase contrast, by time lapse cine-micrography using phase contrast, and after fixation and staining with a variety of stains. There was no obvious difference between cultures taken from any of the embryonic and neonatal stages used.

The following kinds of cells could be distinguished: epithelia, fibroblasts, "small cells" and nerve cells, with fibres. Fibroblasts were the first to appear (fig 6.1), at the periphery of the explants as single cells, often in dense outgrowths.

On the 2nd day of culture, many small round cells, often with microspikes grew out from explants of embryonic and postnatal adrenal tissues (fig 6.2). These cells stained very densely and seemed composed mainly of nuclei with a small amount of surrounding cytoplasm. On the basis of their morphology and rapid movement, these cells are most likely macrophages.

Nerve cells grew out after the 5th day of culturing. They possessed long axons extending along the surface of the culture (fig 6.3).

Multinucleated cells were present mainly at the leading edge of the explants. They were very large, compared to other cells of the explant, with short dendrite-like processes (fig 6.4). They started to appear on the 2nd day and became more obvious after 4 days. They grew out from all embryonic stages studied. Their cytoplasm possessed many vacuoles. From the appearance of some

nuclei (fig 6.5) these cells look as if they become multinucleated from a failure of cell division following nuclear division.

Epithelial cells started to move out of the explants around the 3rd day of culturing (fig 6.6). They were arranged more compactly and moved as a group or column. They sometimes had clear cell boundaries and were smaller than fibroblasts, with round to oval shaped nuclei with several nucleoli (fig 6.6). These cells showed none of the vacuolation expected of cortical cells, nor granules expected of chromaffin cells. Therefore, either dedifferentiation occurred in culture or these epithelial cells were of some other type, possibly endothelium.

The time-lapse cinemicrography method was carried out using small explants of embryonic adrenal tissues. The movement of fibroblasts, nerve cells, "small cells", multinucleated and epithelial cells was observed. The cells looked healthy and moved quite a distance from the explant, but it was difficult to relate these cell to cortical and chromaffin cells. Because of this, these cultures provided no useful results on interactions between corticals and chromaffins.

When small pieces of cultured adrenal tissues were fixed, embedded and then sectioned, cells of embryonic and postnatal stages after various times in culture looked healthy except for some cells at the centre of the explant after 3 days culture (fig 6.7). In the central area, cells were highly vacuolated and had lost contact with their neighbours, opening up spaces between cells. This disorganisation of tissue in the centre of the explants got worse the longer the culture period.

6.2.1.2 Whole and Half Gland Cultures

Whole glands and halved glands were cultured in small petri dishes. The glands were suspended in medium so that they did not attach to the substrate and kept in culture for a maximum of 3 days. The cultures were carried out on 16 and 18 day embryonic mouse adrenals and on 1 week postnatal mouse adrenals. The cultured tissues were fixed in half strength Karnovsky for araldite embedding (1 µm semithin sections), or in Bouin for wax embedding (6 µm thick sections). At the start of culturing, the uncultured half of each halved gland was fixed as a control. When a whole gland was cultured, its twin was also fixed at the start of culturing as a control.

As an alternative method, whole adrenal glands were grafted into the chorioallantoic membrane (CAM) of 7 day chick embryos and cultured for up to 3 days. They were fixed and embedded as above.

6.2.1.2.1 Whole and Half Gland Cultures - Results

Two features of these cultures will be described: (a) the general appearance of the glands; (b) the relative movements of major gland tissues during culturing.

(a). General appearance: The glands did not retain their normal organisation as in *in vivo* even after only 1 day in culture. In general, the cells of whole cultured glands did not look as healthy as the controls. Both cortical and chromaffin cells differed in their appearance and did not look healthy. The main features here were the large number of intracellular vacuoles, and empty spaces between cells throughout the gland except for a thin layer of cells under the capsule (fig 6.8). At the centre of the gland many cells were seen as single, appearing

to have lost contact with their neighbours (fig 6.8). Tissue degeneration at the centre of the gland got worse, the longer the period of culture, but in the 3 days culture period I used, the healthy peripheral area remained healthy (fig 6.8).

In **halved** adrenal gland cultures, the overall results were similar to **whole** glands, except that the cultures were somewhat healthier.

The healthy peripheral zone was a little wider in halved glands than in whole ones (fig 6.9). But at the cut surface, there were extensive signs of tissue damage, presumably as a result of the cutting operation (fig 6.9). This appearance did not change significantly during the 3 days culture period.

When chorioallantoic membrane grafted whole adrenals were sectioned after 3 days, they were well attached to the chorioallantoic membrane, shown by the presence of chick blood vessels and cells penetrating into the gland's capsule (fig 6.10). Much of the centre of these grafted glands was composed of dead cells and empty spaces (fig 6.11). Cells were very loose and seemed to lose contact with neighbouring cells. On the other hand, a peripheral layer 5-6 cells deep looked healthier than the rest of the gland (fig 6.11); and some cells in this area possessed lipid droplets within their cytoplasm.

(b) The relative movements of gland tissues during culturing: The rationale behind the whole and halved glands culture experiment was to test whether cortical, capsule and chromaffin tissues were able to move relative to one another, and whether they followed the predictions of Steinberg's differential adhesion hypothesis (DAH). The possible results from this experiment are shown in figure 6.12.

Unfortunately, the overall degenerative changes in the cultured glands made interpretation of possible tissue movements difficult. The culturing of complete glands was intended as a control to assess the effect of culturing the gland; but complete glands, as described, showed extensive degenerative changes, particularly in the medulla whether cultured in dishes or on chorioallantoic membrane. It was therefore not possible to assess the degree of damage caused directly by cutting.

However, it was possible to detect some changes in gland organisation that occurred only in halved glands in culture. These were localised thickening of the capsule (fig 6.9), and spreading of capsule fibroblasts over the cut surface (fig 6.9).

Of 9 halved glands cultured (3 each of 16 and 18 days embryonic and 3 of 7 days postnatal; one gland from each stage cultured for 1 day, 2 days and 3 days). Spreading of fibroblasts was seen in all glands cultured for 2 or 3 days, but not in those cultured for only 1 day, irrespective of the stage of development of the gland. Localised thickening of the capsule was seen in most prominently in 7 day postnatal glands cultured for 2 or 3 days, and to a lesser extent in embryonic stages cultured 2 or 3 days.

The localised thickening of the capsule was several cells thick and was seen on the opposite side to the cut surface.

After 2 or 3 days in culture, there was on the cut surface a single layer of flattened cells. After 2 days, these had covered approximately one third of the cut surface and after 3 days, about two thirds. Since this layer was continuous with the capsule, and appears to extend progressively from the capsule, it is likely that these cells were capsular fibroblasts, or perhaps endothelial cells from capsular blood vessels. I did not carry

out SEM observations on the cut surfaces to confirm the identity of these cells.

There was no sign of cortical cells (which remained healthy in appearance beneath the capsule) spreading -to enclose the medullary cells exposed by cutting the gland.

6.2.1.3 Short Term Culture (2 hr)

16 day mouse embryo adrenals were used in this experiment. The gland were cut into halves and each half was drawn (after cut) using a camera-lucida. The halves were then incubated for 2 hr and then redrawn.

The aim here was to look for any short time changes that might occur, such as if the capsule had a contractile effect (after cutting) on pulling the enclosed adrenal tissues outward or in any other way.

The results obtained are represented in figure 6.13. As can be seen, there were occasional minor changes in halved gland shape after 2 hours culturing, but they were not consistent.

Fig. 6.1. Fibroblast outgrowth from an explant of 17 dme adrenal grown on glass coverslip (arrows), 1 day in culture. (K&Tb). (Bar=100 μm).

Fig. 6.2. A 17 dme adrenal tissue culture grown on glass coverslip showing "small cells" with microspikes after 2 days in culture (arrows heads). (K&Tb). (Bar=50 μm).

Fig. 6.3. A 16 dme adrenal tissue culture grown on glass coverslip showing nerve cells with long axons (arrows) after 5 days in culture. (K&Tb). (Bar=100 μm).

Fig. 6.4. A 16 dme adrenal tissue culture grown on glass coverslip showing multinucleated cells (*) with short dendrite-like processes (arrows) after 2 days in culture. (K&Tb). (Bar=50 μm).

All of the above cultures were explants from small parts of the adrenal.

dme=day mouse embryo, K=Karnovsky, Tb=toluidine blue

Fig.6.1.

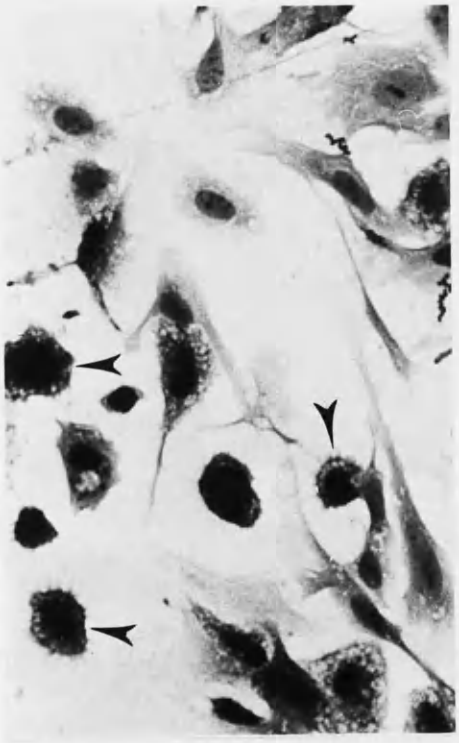


Fig.6.2.

Fig.6.3.

Fig.6.4.

Fig. 6.5. A 16 dme adrenal tissue culture grown on small plastic dish showing a multinucleated cell with nuclear division (arrow' head). after 5 days in culture. (K&Tb). (Bar=50 μm).

Fig. 6.6. A 17 dme adrenal tissue culture grown on glass coverslip showing epithelial like-cells (arrows) with round-oval nuclei with several nuclei after 3 days in culture. (K&Tb). (Bar=100 μm).

Fig. 6.7. Transverse section through 18 dme adrenal small explant culture grown on glass coverslip showing cell death among explant cells (arrows) after 3 days in culture. Cell death is recognised by the presence of vacuolated cells and empty space between cells. Arrow indicates mitotic figure. (K, Ar&Tb). (Bar=50 μm).

Fig. 6.8. Transverse section through whole gland culture of 18 dme adrenal floating in plastic dish showing unhealthy central part with healthy periphery after 2 days in culture. The capsule is indicated by X. (K, Ar&Tb). (Bar=100 μm).

Ar=araldite resin, dme=day mouse embryo, K=Karnovsky, Tb=toluidine blue, X=capsule

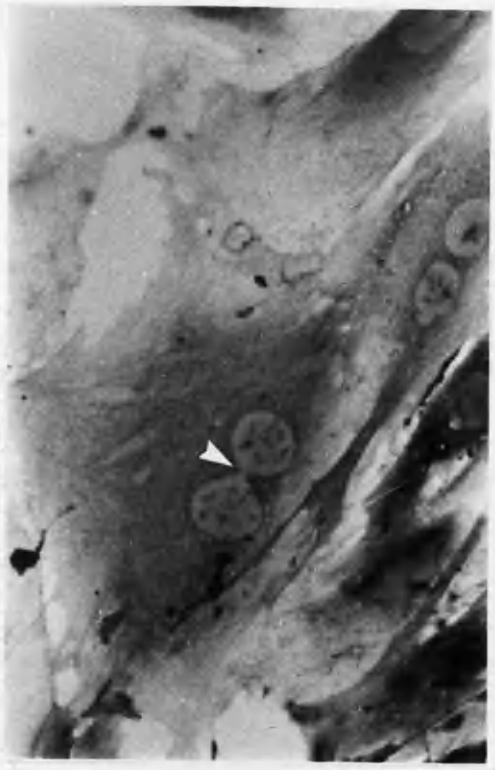
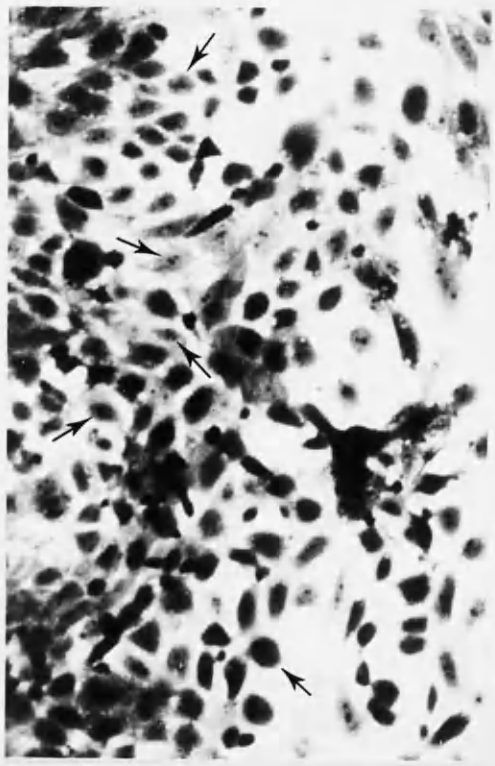


Fig.6.5.



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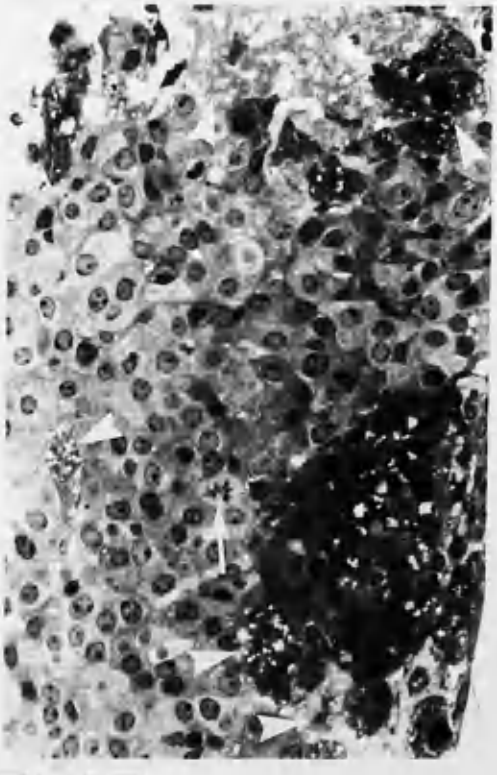


Fig 6.7.

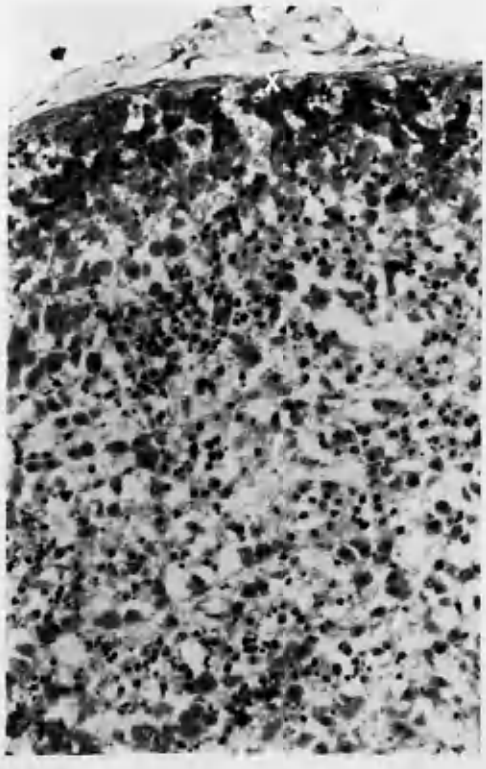


Fig.6.8.

Fig. 6.9a. Transverse section through 1 wpm half adrenal gland cultured floating in small plastic dish for 3 days, showing cell death within both cortical and chromaffin tissue, thickening of the capsule (X) and single layer of fibroblast cells (arrows) starting to appear around the cut surface. Cut surface to the left. (K, Ar&Tb). (Bar=100 μm).

Fig. 6.9b. An enlargement part of Figure 6.9a showing thickening of the capsule (X). (Bar=50 μm).

Fig. 6.10a. Transverse section through 1 dpm adrenal cultured on CAM, after 3 days, showing chick blood vessels and cells (arrows) penetrating the mouse adrenal gland's capsule (X). (K, Ar&Tb). (Bar=50 μm).

Fig. 6.10b. An enlargement of Figure 6.10a showing chick blood cells (arrows) within the capsule. (Bar=25 μm).

CAM=chorioallantoic membrane, dpm=day postnatal mouse, wpm=week postnatal mouse, X=capsule

Fig.6.9a.

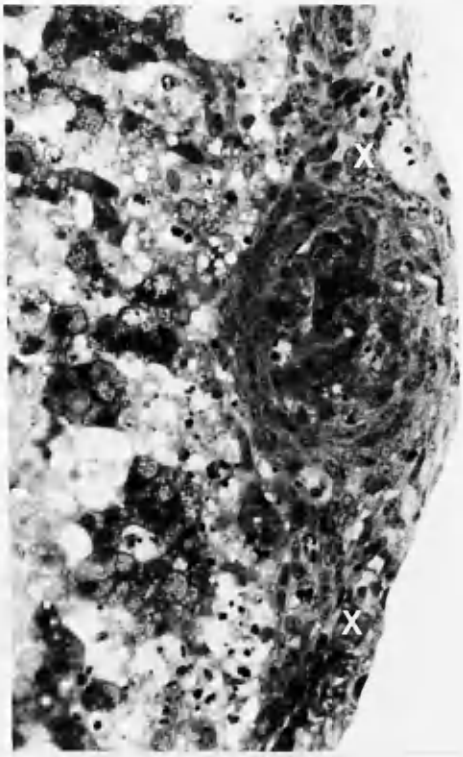


Fig.6.9b.

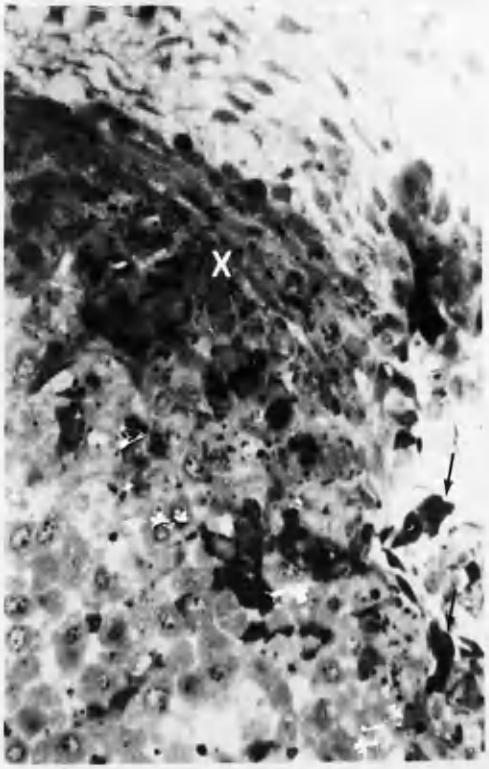


Fig.6.10a.

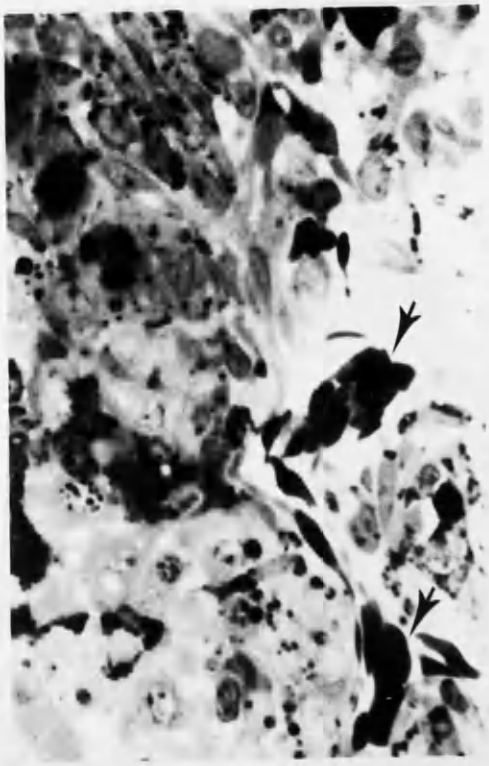


Fig.6.10b.

Fig. 6.11. Transverse section through 1 dpm adrenal cultured on CAM, after 3 days showing cell death among the central parts but healthy peripheral tissue. The capsule is indicated by X. (K, Ar&Tb). (Bar=50 μm).

CAM=chorioallantoic membrane, dpm=day postnatal mouse, X=capsule

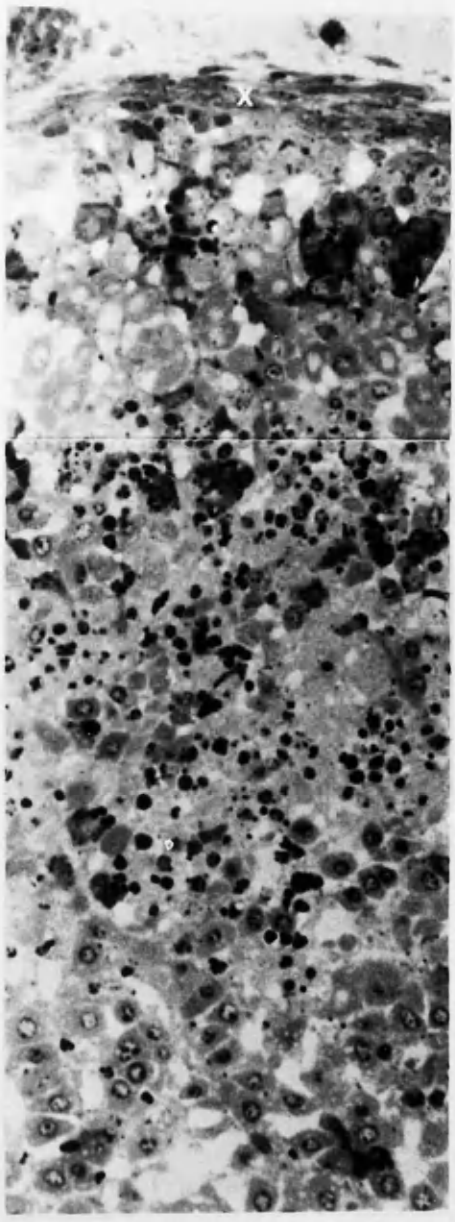


Fig.6.11.

Fig. 6.12. (a). Drawing of halved mammalian adrenal gland showing the anatomy of gland, at the start of culturing. (b). A drawing of one predicted result after culturing a halved adrenal gland, to show cortical tissues surrounding and internaling the chromaffin tissues. (c). A drawing of a second predicted result after culturing a halved adrenal gland, to show a thin layer of capsular cells covering the exposed cut surface.

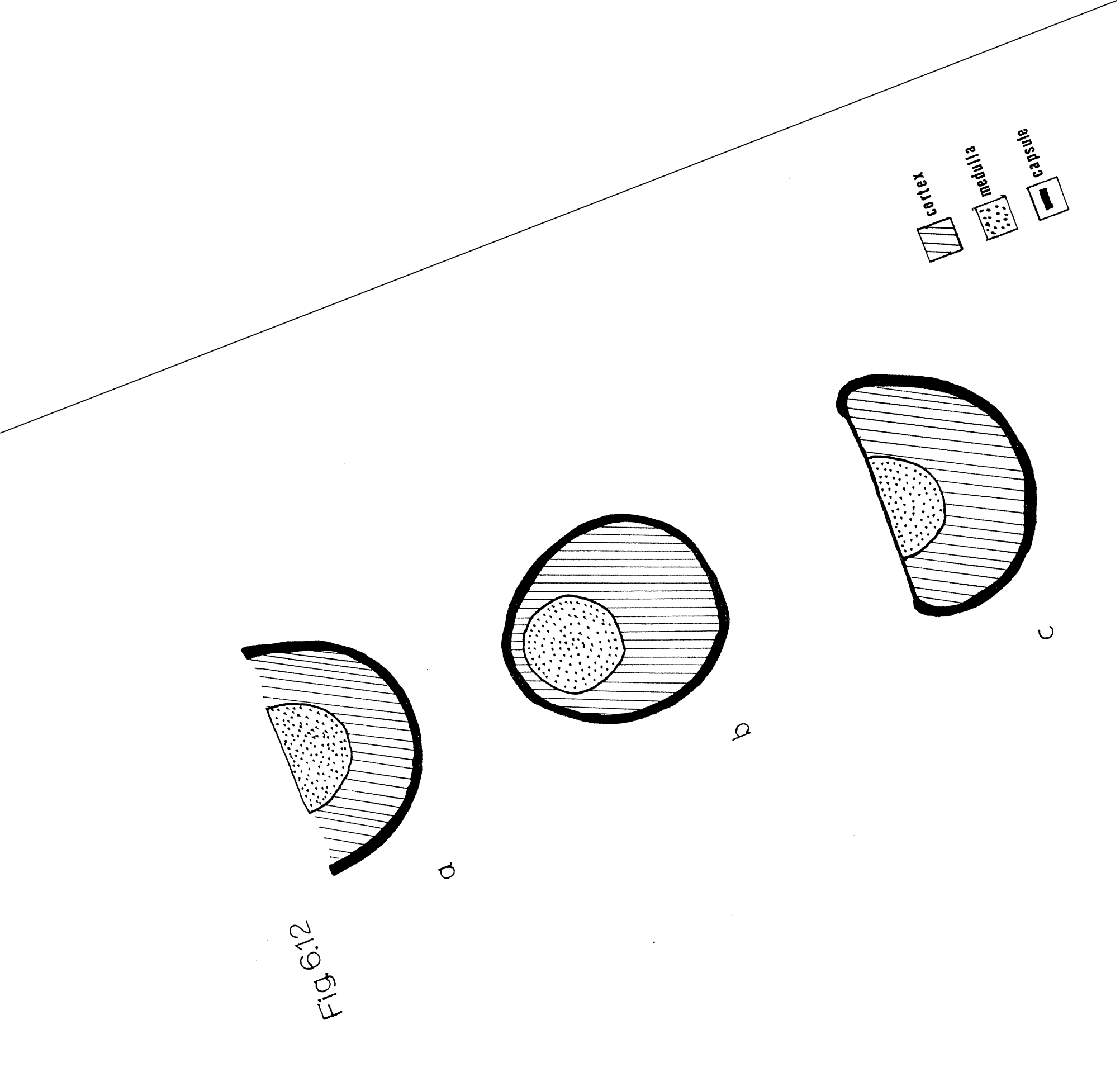


Fig 612

Fig. 6.13a,b,c. Camera lucida drawings of short term culture (2 hr) of halved adrenal glands showing samples of the results obtained. The cut surfaces are to the right.

Solid line=before culturing

Broken line=shape after 2 hr of culturing

Fig.6.13a

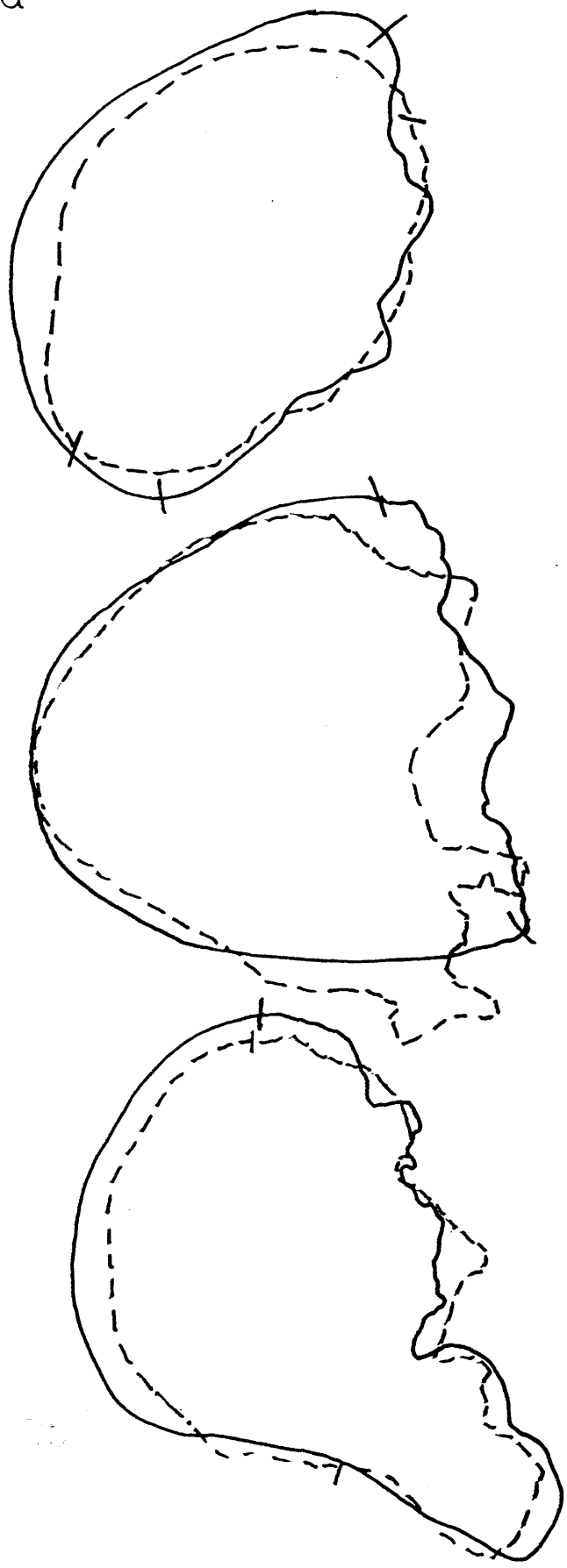


Fig. 6.13b

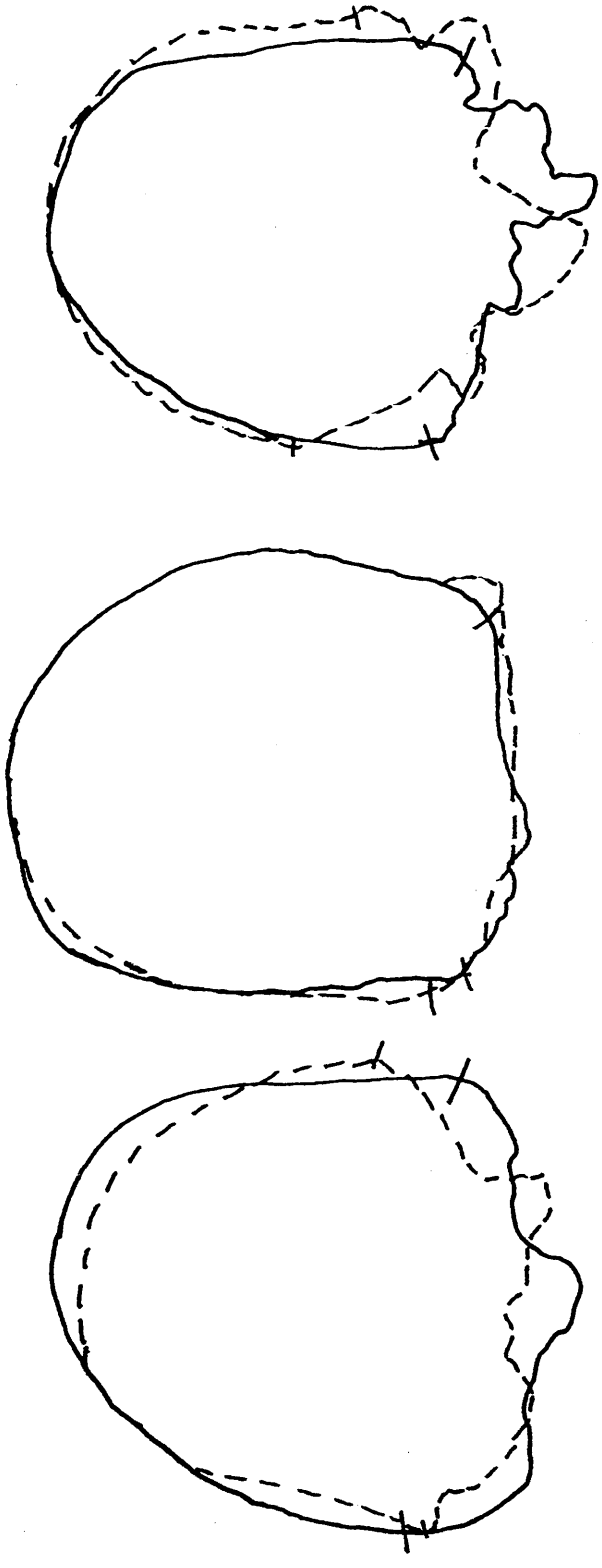
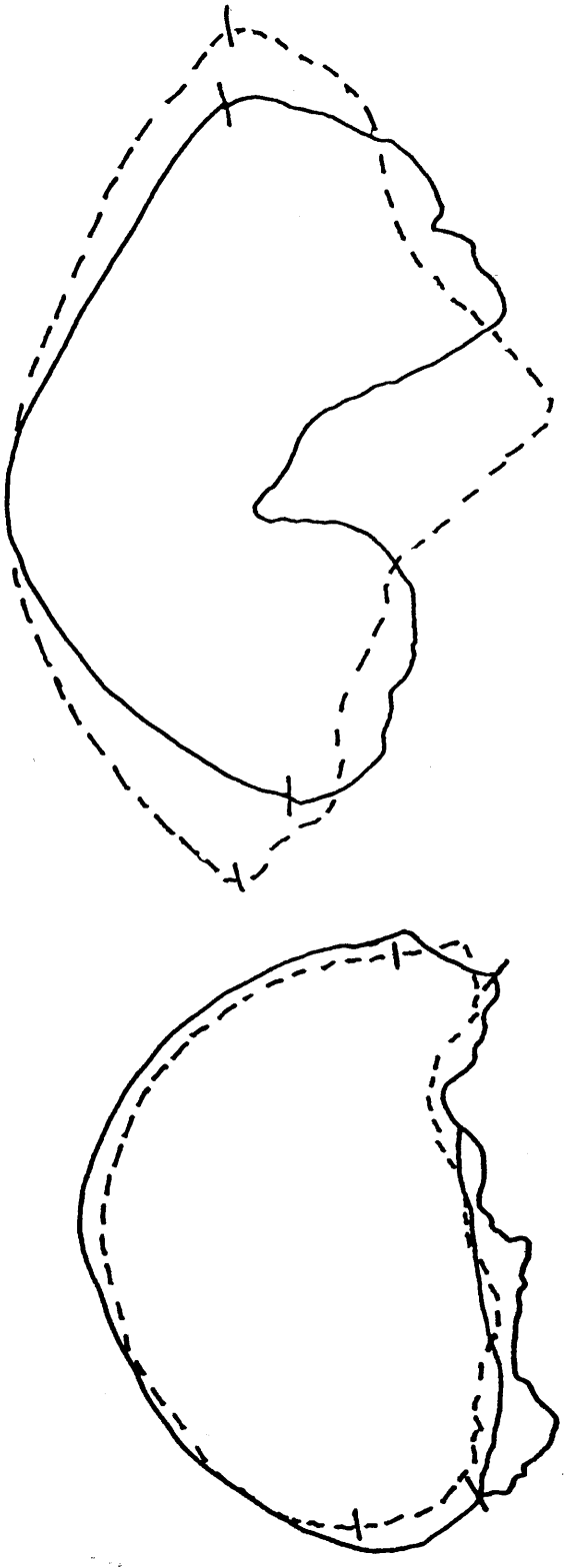


Fig.6.13c



6.3 Discussion

The present work was done mainly on embryonic, postnatal tissues (up to 7 days) and some adult tissues. The embryonic and early postnatal tissues were easy to maintain in culture, whereas the adult tissues were not, therefore the adult tissues were discarded and hence are not included in this discussion. I will discuss here two aspects of my culturing results: (a) culture on flat substrates and observation of cell interactions, (b) culture of whole and halved glands.

6.3.1 Culture of Small Pieces of Adrenal Tissue on Flat Substrates

Small explants of adrenal tissues were kept in culture up to 15 days. The overall appearance of the migrating cells was good. Cells maintained the same shape and characteristics as those seen in the shorter period cultures.

When explants were fixed, embedded and then sectioned, signs of degeneration were seen after 3 days culture within the explant, though not in the outgrowth. Kahri (1966) claimed that no degeneration occurred in his small piece cultures of fetal (19-21 day) and newborn rat adrenal tissue in the course of 3 weeks culture, but that a slow degeneration occurred in longer term cultures. Kahri (1966) noted that if explants were too large a central necrosis occurred and outgrowth was not so good.

The difference between my results and those of Kahri (1966) may be due to the different media used. Kahri (1966) used 50% Melanick's A, 25% Parker's amino acid and calf serum, whereas my culture medium was ham with fetal calf serum.

Several types of cells were seen in my culture outgrowths: epithelia, fibroblasts, multinucleated cells, "small cells" and

nerve cells with fibres. I believe that the epithelial cells may represent both chromaffin and cortical cells, but it is hard to be sure, and I was unable to distinguish the two main cell types in culture. Kahri (1966) showed that the epithelial cells present in his culture outgrowths stained positive for steroid dehydrogenase, and hence that they corresponded to cortical cells. His finding was confirmed by TEM work.

The fibroblasts may represent the mesenchymal cells which migrate from the capsule and blood vessels. Multinucleated cells were very large but few in number and I believe that they derived from fibroblasts. Using cine micrography, the "small cells" showed very rapid movements and hence I believe that these cells were macrophages.

I had hoped in my cultures to be able to distinguish the cortical and chromaffin cells, and to study their interactions, and thereby gain some insight into how these cells form the cortical-medullary pattern. Clearly, different procedures will be needed to study this problem.

Microsurgery to separate adrenal tissues might yield good results. This procedure might work in the postnatal tissues (after 7 days) when the two tissues are quite distinct from each other and can easily be dissected out from each other. On the other hand, the problem of embryonic tissues is that tissues are still intermingled with each other. In fact, I did attempt microsurgery to separate the two tissues in postnatal stages, but was not sufficiently successful.

Another approach to the problem might be the disaggregation of adrenal tissues with culturing in micromass systems similar to the work of Wedden *et al* (1987) on cartilage differentiation of

chick facial primordia. The problem here again is that the different cell types would not be distinguishable after some time in culture.

Other possible method is to find a specific substrate that might encourage the migration of one cell type rather than the other, similar to that used by Kosher & Rodgers (1987). Their work separated myogenic and chondrogenic progenitor cells from one another in chick embryo limb buds (stage 20-22). It would be worth while to try and find something similar for adrenal tissues.

6.3.2 Whole and Halved Gland Culture

This section will discuss three aspects of my whole and halved adrenal gland cultures: (a) Health of tissues in longer term cultures. (b) Relative cell movement in halved glands. (c) Short term (2hr) changes in shape of halved adrenal glands.

(a) **Health of tissues in longer term cultures.** The overall appearance of the cultured glands was not good. Both cortical and chromaffin cells showed cell death in halved and whole glands, cultured and grafted ones. The results showed that degeneration is not the result of cutting the glands in half.

The peripheral parts of the cultured glands looked healthy, compared to central parts and cut surfaces. The reason could be that the outer cell layers were better nourished and oxygenated than the inner layers. Baker & Carrel (1939) showed that a considerable portion of the adrenal gland degenerated after a few days in culture. Mortinovitch (1955) also indicated that a large portion of the central part of a rat (4-5 day old) adrenal cortex underwent rapid degeneration, and only those cells bordering the capsule and a part of the medulla appeared healthy. Schaberg

(1955) also showed that the central parts of 2 day old rat adrenal glands (cut into 5-8 pieces) underwent rapid degeneration whereas the peripheral parts were healthy. Trowell (1959) indicated that when a whole gland of a 4 weeks old rat was cultured, the inner 2/3 of the cortex become entirely necrotic within 2 days, but stated that the outer cortex and medulla remained healthy for 9 days, and that even when a 1/2 or 1/4 gland was cultured the same results were seen.

Mortinovitch (1955) and Trowell (1959) both indicated that the dead part of the cortex was the zona fasciculata and Mortinovitch (1955) argued that the zona reticularis also showed necrotic signs.

Baker & Carrel (1939) and Mortinovitch (1955) claimed that degeneration was brought about by an insufficient supply of oxygen, and Mortinovitch (1955) suggested that cortical cells are more sensitive to oxygen supply than chromaffins.

Trowell (1959) showed that the addition of ACTH and ascorbic acid made no useful contribution to the health of adrenal gland cultures. He found that when the gland is submerged in medium, a large central necrosis occurs. Therefore when culturing glands, he suggested that they must be supported on a grid so that they project well into the gas phase, to allow an adequate uptake of oxygen.

Aramato & Nussdorfer (1972) showed that when adult rat adrenal glands were decapsulated and their zona glomerulosa removed, then the remaining adrenals cut into quarters, larger explants underwent necrosis within 3-5 days, but the peripheral layers remained healthy. They showed that necrosis did not occur in smaller explants.

We can conclude that the capsule of the gland prevents nutrition and oxygen from getting through to the inner tissues of the cultured gland. Also, there may be a need for a medium supplementation, possibly hormones. Improvements in culturing technique might therefore allow a more successful attempt at the halved gland experiments I have described.

(b) **The relative cell movements in halves glands.** There were two changes in the shape of the cut glands (2 and 3 days cultures) when compared to the other cut gland (same gland) which acts as a control. The first change was the appearance of a single layer of fibroblasts at the edge of the cut side. These fibroblasts seem to migrate from the capsule over the cut surface, and represent some sort of wound-healing process. The other change was the thickening of the capsule on the opposite side to the cut surface. This may be due to contraction forces in capsule fibroblasts made possible by the cutting open of the gland. There was, however, no sign of the cortical tissues spreading to internalise the medullary tissue.

The significance of these results in the light of the differential adhesion hypothesis will be discussed fully in chapter 8.

(c) **The short term changes in shape of halved glands.** The aim here was to demonstrate whether the capsule might be exerting force on the inner part of the gland, by cutting the gland in half, then observing any changes in shape that occurred over the next few hours. If capsule contraction occurs, a cut gland would be expected to change shape as in figure (6.13). As the adrenal gland develops, the capsule becomes thicker, and the internal tissues become more compact. It is possible that these two processes are related: the capsule may not be simply a barrier,

but also contractile, compressing the interior of the gland. Harris *et al* (1981) and Oster *et al* (1983) have shown that fibroblasts do exert tractional forces on collagen fibres of the extracellular matrix. The overall results in my experiments showed no dramatic changes occurred in gland shape; there was no sign of the capsule pulling the inner tissues apart. This is evidence against capsule contraction, but is not conclusive. It could be that contraction occurred too quickly to be observed, at the time of cutting, or it could be much slower and therefore affected by the degenerative changes that occurred with longer term culturing. Furthermore, if there was an internal force exerted on the gland, it might be lost while the cutting took place. Under these conditions, the capsule might not be able to pull the inner tissues apart. As discussed in (b) above, the thickening of the capsule opposite the cut surface in halved glands cultured for 2-3 days may be evidence for capsular fibroblast contraction.

CHAPTER SEVEN

Quantitative Analysis of the Mouse Adrenal Gland

7.1 Introduction

Morphometric analysis of histological sections as a means of determining shapes and dimensions of component tissues has been used for many years. Until recently, it has usually been necessary to employ relatively crude methods for estimating shape and size but increasingly, computer programmes are becoming available that allow both more accurate measurements to be made, and more sophisticated analysis to be carried out on these measurements. Berkwits & Iannaccone (1985) developed a method for doing quantitative analysis of irregular areas such as histological sections of chimeric tissue in rat liver, by using computers. A recent review of such methods is by Huijsmans *et al* (1986).

Previous morphometric analysis on adrenal gland has been carried out by Donaldson (1919), who determined the relationship of the cortical and medullary tissues in the rats and demonstrated changes in their volume with age. Baker (1937) measured the weights of the adrenal glands of mature and immature male and female dogs. Shire & Spickett (1968) determined which part of the adrenal was responsible for the quantitative differences in adrenal weight between neonatal and young adult mice of different genotypes.

Several methods were used in these previous studies, such as the planimeter and weight method (Baker, 1937), projection, enlargement and tracing sections with a planimeter (Donaldson,

1919 and Shire & Spickett, 1968).

The aims of this part of my study are to do a quantitative analysis of morphogenetic aspects of the mouse adrenal gland at different stages of development. The aspects I considered are:

I) Growth of the different components of the gland -cortex, medulla -in terms of dimensions (volume, tissue width) and cell proliferation (mitotic index).

II) Cell death - distribution and numbers of dying cells in different parts of the gland at different stages.

III) The position of the medulla within the gland - is it central, or off to one side; constant or variable.

7.2 Results

7.2.1 Growth of the Different Components of the Gland

7.2.1.1 Size Measurements of Gland Components

Measurements were made on 1 μm semithin araldite sections stained with toluidine blue of the following mouse adrenal gland stages (15, 17 day embryos and 1 week postnatal).

7.2.1.1.1 The Volume and Ratio of Adrenal Tissues

During embryonic life the adrenal gland does not have the two distinct regions (cortex and medulla) but consists of groups of cells intermingled with each other (fig 7.1). In contrast the 1 week postnatal adrenal has two distinct regions (cortex and medulla) (fig 7.1).

For method used see chapter 2 (section 2.3).

The results are shown in Table 7.1 and Figure 7.2. The cortical tissues showed considerable growth during embryonic development, whereas the chromaffin tissues showed little growth

during the embryonic period (fig 7.2), but considerable growth during the early postnatal period. The ratio of chromaffin to cortical volume was is 1:8 during embryonic life but 1:4 by 1 week postnatal.

7.2.1.2 Mitotic Counts

Mitotic counts were made 1 um serial sections of 15 and 17 day mouse embryonic adrenals, and 7 day postnatals. Counts were made using a Wild M20 microscope with an objective magnification of X40. In the eye-piece was a square graticule. All cells within a 120x120 μm (at x500 overall magnification) square were counted.

For each series, one section in each group of 6-8 sections was counted. For 15 and 17 day embryos, two areas from each section were chosen randomly, to include both chromaffin and cortical cells. For 7 day postnatals, separate counts were made from cortical and chromaffin areas. Cells were counted where the section passed approximately through the centre of the nucleus.

Results are shown in Table 7.2. The mitotic index was at the highest rate in 15 day embryonic adrenals (cortical=1.61%, chromaffin=1.26%) and declined thereafter.

7.2.2 Cell Death

Counts of dying cells were made in the same way as for mitotic cells. In 15 and 17 day embryos, it is not possible with the light microscope to distinguish between dying chromaffin and cortical cells, but this is possible in postnatal stages when the two tissues are clearly segregated. The results are shown in Table 7.3. I found that cell death was at its highest rate in 17 day embryonic adrenals (5.23%) but in 15 day embryonic adrenals

it was 2.22% and in 7 day postnatals it was 6.1% for chromaffin and 3.2% for cortical tissue.

7.2.4 The Relative Position of the Medulla

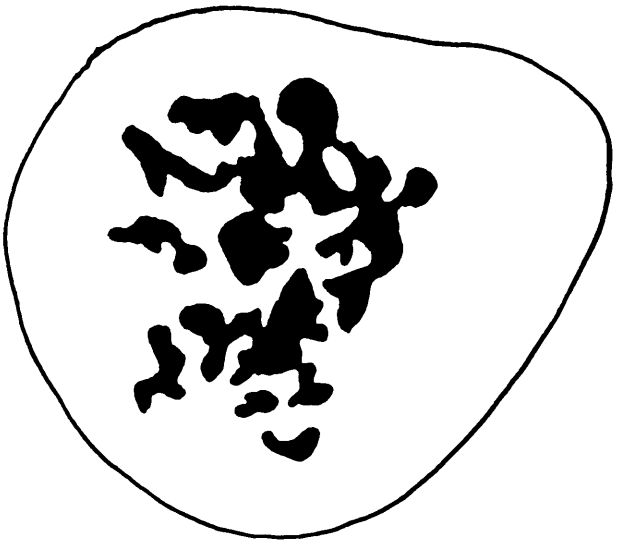
Mouse adrenal glands vary in their shapes and sizes even in the same animal (both embryonic and postnatal); some are triangular or somewhat elongated and others are oval-round. In embryonic specimens the cortical and chromaffin tissues are intermingled with each other (fig 7.1a, b) and this makes it impossible to give the exact position of the medulla at these stages.

To determine the relative position of the medulla as soon as it had completely formed, serial lum sections of 4 and 7 day postnatal adrenals were examined and sections found where the medulla showed the largest area (i.e. sections through the middle of the medulla). Camera lucida drawings through these sections are shown in Figure 7.3 and 7.4, along with measurements of the distances from the medulla to the outer edge of the cortex.

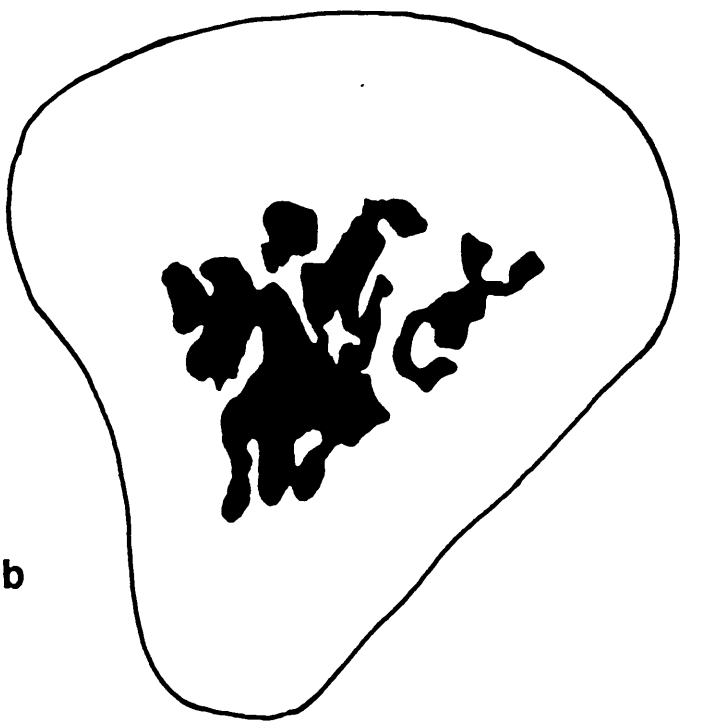
It is clear that the position of the medulla is variable, sometimes very near the centre of the mass of cortical tissue, sometimes off to one side (fig 7.3, 7.4).

Fig. 7.1. Camera lucida drawings of sections through the centre of typical mouse adrenals. a) 15 day embryo. b) 17 day embryo. c) 1 week postnatal. Dark areas represent cortical tissues and light areas represent chromaffin tissues. (Bar=250 μm).

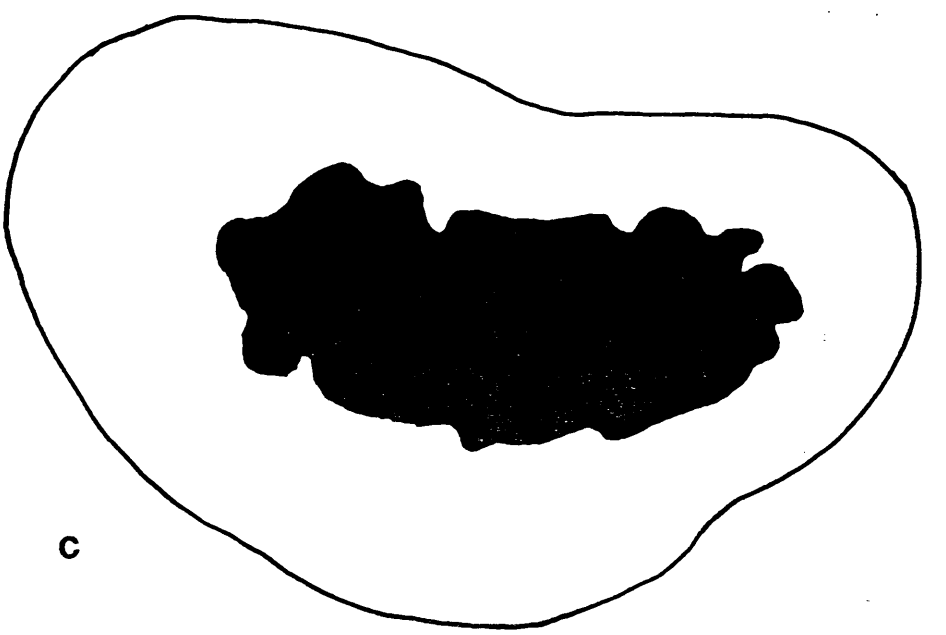
Fig. 7.1



a



b



c

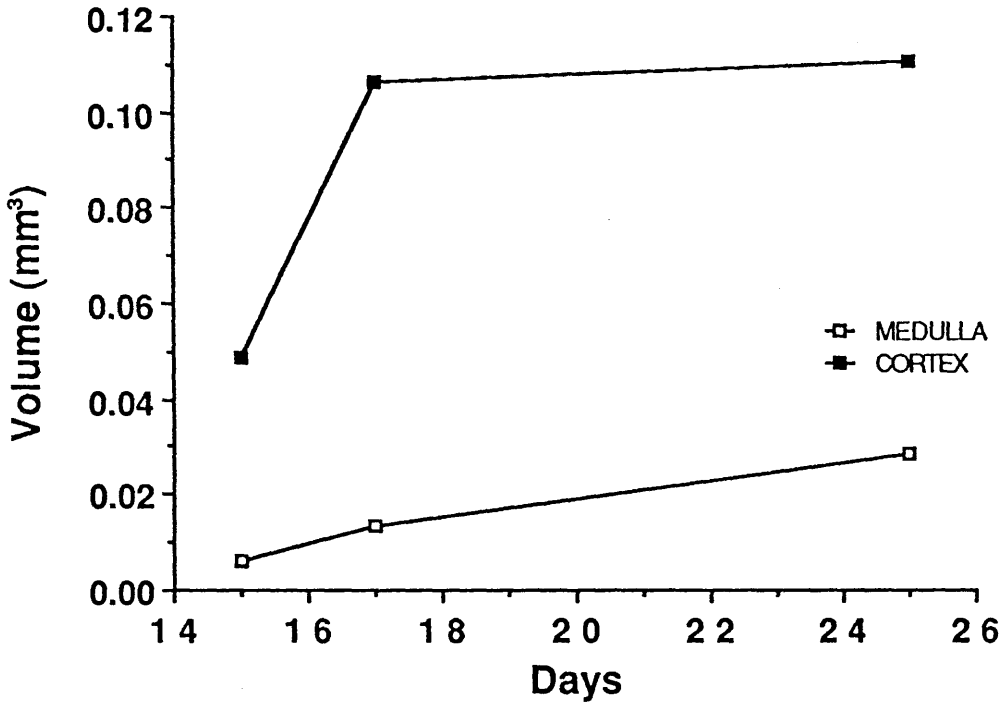
Table 7.1 Volume of Adrenal Gland Tissues

Age	Chromaffin Tissue		Cortical Tissue		Ratio Chrom:Cort
	No.glands measured	Volume (mm^3) mean (range)	No.glands measured	Volume (mm^3) mean (range)	
15 dme	3	0.0062 (0.0086-0.0042)	3	0.0498 (0.0542-0.0426)	1:8
17 dme	3	0.0134 (0.0159-0.0111)	3	0.1066 (0.1291-0.0856)	1:8
1 wpm	2	0.0286 (0.0299-0.0273)	2	0.1110 (0.1213-0.1007)	1:4

dme = day mouse embryo
wpm = week post natal mouse

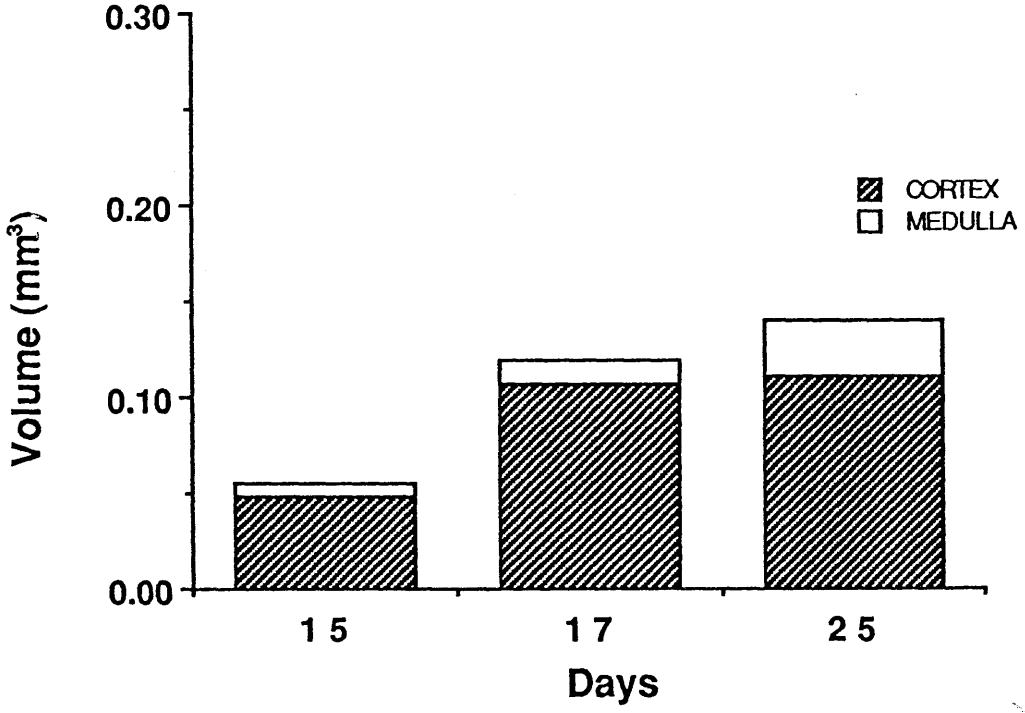
Fig. 7.2

Measurement of mouse adrenal gland tissues



a

15 & 17 days are embryonic tissues
25 days = 7 days post natal tissue



b

Table 7.2 Mitotic Indices of Adrenal Gland Tissues

Age	Chromaffin Tissue				Cortical Tissue			
	No. glands counted	Total cells counted mean (range)	Total mitotic cell count mean (range)	Mitotic index (%) mean (range)	No. glands counted	Total cells counted mean (range)	Total mitotic cell count mean (range)	Mitotic index (%) mean (range)
15 dme	3	827.7 (693-905)	10 (7-16)	1.19 (0.77-2.26)	3	1497 (1221-1660)	24 (18-29)	1.58 (1.11-2.01)
17 dme	3	1151.3 (944-1431)	7 (4-12)	0.60 (0.42-0.83)	3	1657.7 (1429-2083)	12.3 (6-22)	0.74 (0.29-1.48)
1 wpm	2	2010.5 (1991-2030)	7 (6-8)	0.35 (0.30-039)	2	2549.5 (2396-2703)	17.5 (17-18)	0.68 (0.63-0.70)

dme = day mouse embryo
wpm = week post natal mouse

Table 7.3 Dead Cells

Age	No.glands counted	Total cells counted mean (range)		Dead cells % mean (range)			
15 dme	3	2412.3 (1968-2716)		2.22 (0.66-4.23)			
17 dme	3	2971.3 (2567-3718)		5.23 (1.52-9.24)			
1 wpm	2	Chromaffin no. counted mean (range)	Cortical no. counted mean (range)	Chromaffin mean (range)		Cortical mean (range)	
				No dying cell count mean (range)	% mean (range)	No dying cell count mean (range)	% mean (range)
		2010.5 (1991-2030)	2549.5 (2396-2703)	129.5 (61-198)	6.1 (2.9-9.0)	83 (45-121)	3.2 (1.8-4.3)

dme = day mouse embryo
wpm = week post natal mouse

Fig. 7.3a-c. Camera lucida drawings of sections through the centre of 4 day postnatal mouse adrenal glands showing the position of the medulla within the gland and measurements (μm) of its width, length and the distance from the gland's capsule. (Bar=133.3 μm).

Fig.7.3

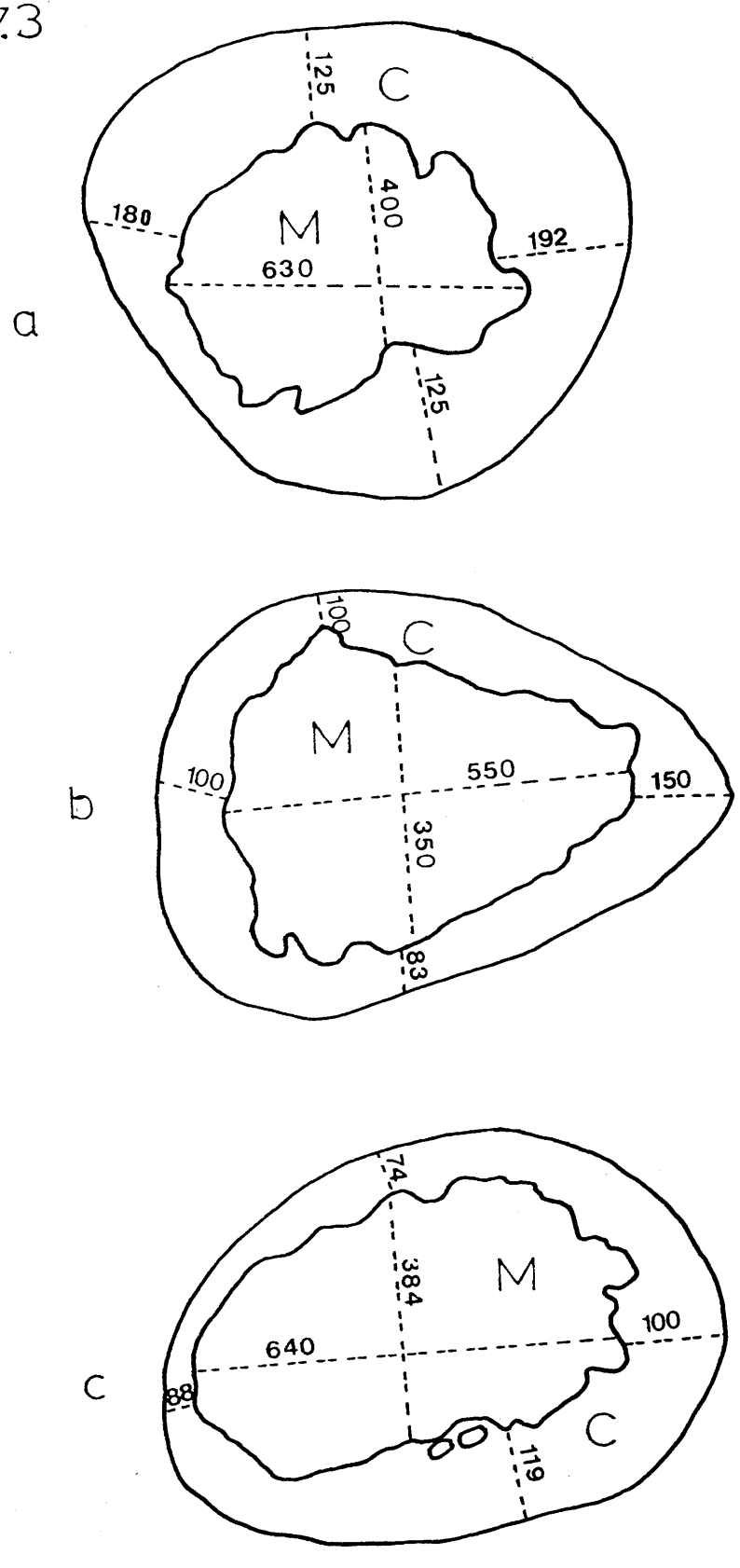
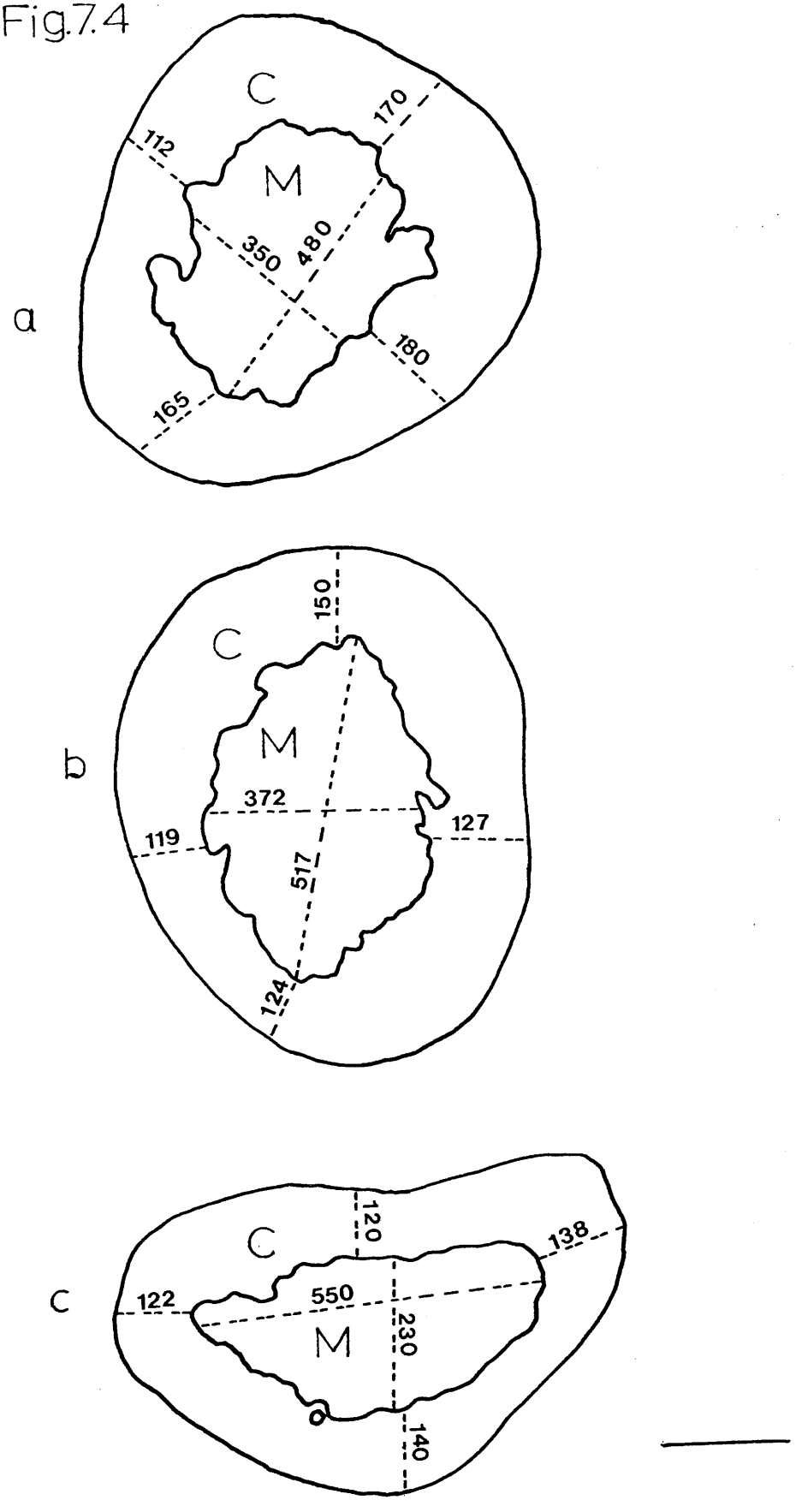

a
125
C
180
M
400
192
630
125
b
100
C
M
100
550
150
350
83
c
74
384
M
100
640
88
C
119

Fig. 7.4a-c. Camera lucida drawings of sections through the centre of 7 day postnatal mouse adrenal glands showing the position of the medulla within the gland and measurements (μm) of its width, length and the distance from the gland's capsule. (Bar=133.3 μm).

Fig.7.4


a
C
M
112
170
350
480
180
165
b
C
M
150
372
119
127
517
124
c
C
M
120
138
122
550
230
140

7.3 Discussion

Measurements were carried out on mouse embryo (15, 17 day) and postnatal (4, 7 day) adrenal glands. The following results will be discussed here: The growth of the different components of the gland (volume and ratio of main adrenal tissues, and mitotic indices), cell death, and the relative position of the medulla.

7.3.1 The Growth of the Different Components of the Gland

7.3.1.1 The Volume and Ratio of Cortical and Chromaffin Tissues

This study showed that the relative volume and ratio of adrenal tissues changes between embryonic and postnatal stages in the mouse.

In embryonic stages, the chromaffin tissue makes up only one ninth of the complete gland, but increases to one fifth by one week postnatal. The postnatal proportion I found agrees well with previous results on adult adrenals: Shire (1970) one fifth, in young male mice and Howard-Miller (1927) one eighth in adult female mice.

There seem to be no previous calculations of relative chromaffin-cortical volumes in the adrenals of the mouse embryo.

7.3.1.2 The Mitotic Counts

It was beyond the scope of this study to make a detailed analysis of proliferation rates in chromaffin and cortical tissues throughout their development. This would be a major task, particularly in embryonic stages when it is not always possible to distinguish whether a mitotic cell is a chromaffin or cortical cell, at light microscope level.

What I hoped to gain from a simple count of mitotic and non-

mitotic nuclei was some general indication of possible changes in proliferation in the tissues at different stages. The mitotic index, particularly when not corrected for cell size differences, cannot provide an accurate measure of proliferation rate.

My data indicate that mitotic index is always higher in cortical than in chromaffin tissue, and that in both tissues, it declines from embryonic to postnatal stages.

One puzzle concerning these results is that from the volume data, the proportion of chromaffin to cortical tissue increases between 17 days embryonic and one week postnatal. It is possible that the reason for this discrepancy is a change in cell size, but I have not measured this feature. The decline in mitotic index is however reflected in an overall decline in volume increase (both cortical and chromaffin volumes double between 15 and 17 days embryonic, but increase much more slowly thereafter).

7.3.2 Cell Death Counts

The overall proportion of dying cells increased during the embryonic period. At one week postnatal, the overall proportion of dying cells is 4.5%, with the rates in chromaffin and cortical tissues being 6.1% and 3.2% respectively. The difference between the two tissues may be artefactual, since at the light microscope level, it is not possible to be sure whether a dying cell is of cortical or chromaffin type, and my counts were made on the basis of the positions of these cells. At one week postnatal, some cortical cells do remain in the medullary area,and it may be that most of these are dying by this stage.

A detailed discussion on cell death and its role in adrenal development is given in chapter 8.

7.3.3 The Relative Position of the Medulla

During embryonic life both cortical and chromaffin tissues of the adrenal are intermingled; it is not until postnatal life that the chromaffin tissue (medulla) is completely segregated. The position of the medulla within the gland is variable, sometimes being very nearly central, sometimes being off to one side. There has not been any previous study on the shape and position of the medulla of the mouse adrenal gland. The relevance of this feature to the method of tissue segregation in the adrenal gland will be discussed in chapter 8.

CHAPTER EIGHT

General Discussion

Since individual chapters have their own discussion sections, this General Discussion chapter will deal with two major themes arising from my work: (a) the role of cell death in adrenal development, (b) the means by which cortico-medullary tissue segregation occurs in the adrenal and its relevance to the Differential Adhesion Hypothesis (DAH) (Steinberg, 1964, 1970).

8.1 Cell Death

An unexpected result of my work was the discovery of cell death among the embryonic tissues in both mouse and chick adrenal glands.

In the mouse embryo, cell death was present in all stages that I studied (14-18 day embryos), and throughout the gland. Electron microscopy confirmed that dying cells were of both cortical and chromaffin type.

In the chick embryo, cell death was also present but in later stages of development (17-19 days), throughout the gland, and again involved both kinds of cells. I could not see any dying cells in the earlier stages examined (15-16 days).

Cell death has been reported in many organs and at many stages in vertebrate embryos, but not before in embryonic adrenal glands. Cell death has several possible functions during development though not all of these are well established, and there are several cases where cell death occurs with no known function.

Cell death plays a role in shaping the form of tissues and organs by removing redundant cells, so that the animal is born with the right organs hence enabling it to survive. Mitrovic (1977) showed that cell death is involved in the formation of embryonic joint cavities. Hogg *et al*(1983) indicated that cell death is important in lumen formation of the mammary gland of the developing mouse by means of separation of cells whose opposing faces are non-adhesive. Schoenwolf (1981), showed that cell death is involved in the remodelling of the tail region of the chick embryo. It is also involved in separating and shaping the avian digits by removing cells from the interdigital areas (Hinchliffe & Johnson, 1980).

Cell death may play a role in the elimination of imperfectly matched cells, particularly in the nervous system. Hamburger (1975) showed that cell death is present in the lateral mater column of the chick embryo where axons that failed to compete with others at the periphery and make connections were eliminated. Landmesser & Pilar (1976) showed that cells of the nervous system compete for peripheral synoptic connections and that those which are capable of making connections survive while others that form an insufficient number of synopses die.

Cell death may also be involved in the elimination of migratory cells that do not reach their correct destination, as in the case of primordial germ cells. It is certainly known that some primordial germ cells fail to reach the gonadal ridges but their later fate has not been well established (Zamboni & Upadhyay, 1983).

Cell death is a normal end-point of some kinds of organogenesis and histogenesis such as long bone formation where cartilage cells die and are replaced by marrow and bone, or in X-

zone degeneration in mouse adrenal gland, where a cell layer degenerates to form an inner capsule separating the medulla from the cortex.

Another function of cell death during development might be to create space where cells can move around. Glücksmann (1951) stated that cell death is found during embryogenesis and that it is abundant wherever the regular arrangement and close packing of cells prevents free cell movement. Mitrovic (1977) suggested that the space created by cell death when embryonic joint cavities are forming might be used by blood vessels to pass through those areas. Cuadros & Rios (1988) showed that cell death is present in the retina of the chick and that cell death might play an important role in setting up the initial pattern of optic fibre growth.

However it is not always obvious why cell death occurs. Wyllie *et al* (1973a) found cell death in normal neonatal rat adrenal cortex mainly in the inner part of the cortex at 1-49 days. They suggested that cortical cell death is the result of lowered ACTH secretion in the neonate, but could find no functional significance for this outbreak of cell death. Hinchliffe & Johnson (1980) showed that although cell death is involved in shaping the chick limb, it is not obvious why it happens in certain areas, such as the posterior necrotic zone (PNZ), anterior necrotic zone (ANZ) and the opaque patch.

In the adrenal gland of the mouse embryo, cell death was at its highest on the 17th day of gestation. By this time a large number of chromaffin cells are moving to the centre of the gland to form the future medulla and cortical cells are moving out of the central area. When a cell dies, it leaves an empty space

behind. I believe that this empty space may allow living cells to move about, hence chromaffin cells are using these spaces to move into the centre and cortical cells into the periphery. It is also possible that cortical cells use these spaces for overall future rearrangement into zones, and that blood vessels and nerve fibres use these spaces to reach their targets within the gland. Since the distribution of cell death is throughout the gland, it cannot be that individual spaces created by dying cells are immediately used by adjacent migratory cells. Rather, the overall looseness of the gland created by cell death allows more easy relative tissue movements than would occur in a more compact tissue. If this idea is correct, the incidence of cell death ought to relate to the periods of most active cell movement, vascularisation and innervation.

In the chick, the empty spaces which were created as the result of cell death might be used by blood vessels and nerve fibres to reach their targets within the gland, but I could not see any role for cell death in the overall organisation of the adrenal, since no distinct central region (medulla) is present. Several terms have been used to describe different modes of slow cell death; these include cellular degeneration, necrosis, pycnosis and apoptosis. The term necrosis is used to describe cell death which may be caused by external damage and this may affect groups of cells or part of a structure or an organ. Pycnosis is used to describe the morphology of dying cells where the nucleus shrinks and chromatin condenses. Apoptosis was used by Kerr *et al* (1972) and Wyllie *et al* (1980) to describe dying cells characterised by loss of contact with neighbours, cytoplasmic and nuclear condensation and fragmentation but with most of the internal membranes remaining intact. Apoptotic bodies

show degeneration only if ingested by phagocytic cells.

In my study, at the light microscope level, dying adrenal gland cells stained very dark with very vacuolated cytoplasm and irregular dark condensed nuclei. At TEM level, dying cells were characterised by dark staining with very dark irregularly shaped nuclei some of which were shrunken and folded. Some of the cytoplasmic organelles such as mitochondria had a bizarre appearance but retained their membranes. Dying cells generally appeared to retain contact with their neighbours in both mouse and chick embryos and were not rounded up, whereas Wyllie *et al's*(1973a,b) "apoptotic bodies" in the rat neonatal adrenal were rounded up, and had lost contact with their neighbours.

Bellairs (1961) showed that dying cells are characterised by the condensation of chromatin in the nuclei, very dense cytoplasm and the presence of abnormalities of some cytoplasmic organelles, such as mitochondria. Bands of cytoplasmic granules arranged in regular arrays are also characteristic of dying cells (Bellairs, 1961).

There are several ways by which dying cells are eliminated. Most of the above studies showed that macrophages were present in the area of cell death (Sanders *et al*, 1986; Schoenwolf, 1981 and Ballard & Holt, 1968). Beaulaton & Lockshin (1982) showed that elimination of dying cells is either by specialised macrophages or by non-specialised phagocytes such as neighbouring cells which change into macrophages to eliminate the dying cells then return to their normal state.

Wyllie *et al* (1973a, b) found that the removal of neonatal rat adrenocortical dying cells was by means of "histiocytes" (local macrophages) or by sinusoid-lining cells, or possibly by

the passing of dead cells into sinusoids where they could be removed by the blood stream. They ruled out the change of neighbouring cells into macrophages.

In my study there was no sign of neighbouring cells changing into macrophages nor of specialised macrophages. It may therefore be that dying cells were eliminated by means of the circulatory system, as suggested by Wyllie *et al*(1973a,b). A problem with this suggestion is that I have not seen rounded-up dying cells in the embryonic adrenal.

One of the main problems with cell death is to know how long it takes a cell, once it has developed a "dying" morphology, to be eliminated from the population, particularly when it is possible that new dying cells are being added to the population over a period of time. One method that could give useful information on cell death dynamics in the adrenal would be to make careful counts of the dying cell numbers in a large sample of adrenals during embryonic development. Any changes in numbers would indicate recruitment and elimination of dying cells. Unfortunately, if numbers are constant, it could mean an even rate of recruitment and elimination. Without a marker for cell death initiation or elimination, this problem is impossible to solve. My results on dying cell numbers (for details see chapter 7, section 7.2.2) suggest recruitment and elimination are occurring, but the sample size is too small to make definite conclusions.

8.2 Sorting Out of Adrenal Tissues

Adrenal gland development involves two main tissues: cortical and chromaffin. Chromaffin tissue is derived via sympathetic nerve ganglia ultimately from the migratory neural

crest cell population. Cortical tissue is derived from the same sort of tissue as the kidneys and gonads, that is the epithelial covering and underlying mesenchyme of the roof of the body cavity in the trunk region.

In mouse, once chromaffin cells have intermingled with cortical cells, they eventually form a more or less centralised, single mass, the medulla. In chick, chromaffin cells form a series of large or small patches dispersed throughout the gland. I wish to discuss here the possible morphogenetic means by which these patterns could be generated. A survey of the literature suggests the following possible mechanisms: chemotaxis, contact guidance and differential adhesion (for review, see Trinkaus, 1984) and self assembly, recently suggested by McLachlan (1986). Of these, the only one to predict the different patterns found in mouse and chick is differential adhesion (Steinberg, 1964, 1970). Steinberg shows that where the more cohesive cell type is considerably in the minority during the sorting out process, it will form a series of disconnected patches scattered amongst the less cohesive cell type. Where the proportions are more even, it is more likely that all the patches will join up to form a single mass entirely surrounded by less cohesive cells, but this mass will rarely be precisely in the centre.

In the following discussion of different mechanisms, I will assume it likely that mouse and chick adrenal glands are variants on a common morphogenetic mechanism, rather than being entirely different. This assumption could be challenged, but seems to me the most parsimonious basis for the comparison.

The general pattern, of slow and irregular segregation of the adrenal gland cell types, is highly compatible with the

differential adhesion hypothesis (DAH), and much less so with more deterministic mechanisms such as chemotaxis, contact guidance or adhesive gradients.

In the case of chemotaxis, different concentrations of chemical agents need to be present within the aggregate so as to influence the migrating cells, either towards or away from the point of the highest concentration. If the concentration was high in the centre, produced by cortical cells in the centre, I would expect chromaffin cells to migrate individually at an even rate, and directly to the centre; such a pattern of accumulation is not seen in the mouse adrenal and it is not at all clear in such an arrangement what would happen to the central 'producing' cells at the end of the process. In the chick, chromaffin cells are found throughout the gland and do not show concentration in any particular area. Therefore I can say that chemotaxis is very unlikely to be involved in the sorting out process of adrenal gland tissues.

In contact guidance, cells follow physical guide-lines on the substratum on which they are moving. The guide lines may be extracellular matrix fibres, or complete orientated cells. This mechanism would seem to require a fixed substratum, and to be quite unsuitable for a situation where two cell types are moving in opposite directions, using each other as locomotory substrata.

An adhesive gradient suffers from similar problems. In the case of the mouse adrenal, an adhesive gradient could attract chromaffin cells to the centre, but it is hard to see why cortical cells should move in the opposite direction. In the chick, no such gradient could be present. I investigated one possible source of an adhesive gradient - the distribution of fibronectin in the gland. The results showed that fibronectin was

present in both mouse and chick embryonic adrenals, but there was no evidence for a gradient.

"Self-assembly" is another proposal for the generation of cell patterns (McLachlan, 1986). The process of self-assembly involves the co-operative activities of cells of the same type, forming themselves into particular arrangements on the basis of differentiated regional properties of their surfaces or cytoskeleton. There is nothing in principle against such self assembly processes involving more than one type of cell, and being in the same time responsible for tissue segregation (sorting out). However, in the case of the adrenal gland, it is clear that the arrangement of cells into zones and cords occurs when sorting is more or less complete. If sorting itself was brought about by the same regional properties as zonation and cord formation, we would expect to see zones and cords forming while sorting was still occurring. Since this does not happen, it seems reasonable to suggest that cell segregation occurs by a somewhat different mechanism from the later zonation and cord formation, though the later process could well involve some specialisation of the earlier.

The DAH states that when a mixture of two different cell types is placed together a sorting out process takes place in which the more cohesive tissue sorts out internally, not always exactly at the centre, and the less cohesive externally (Steinberg, 1964, 1970). The differences in the relative numbers of the two cell types can affect the final pattern: if the more cohesive tissue is less in number than the less cohesive, then the more cohesive tissue will not form a single mass at the centre, but rather form a number of small patches or groups

intermingled with the less cohesive cells.

A problem with much of the work on the DAH is that most experimental studies have been carried out on artificial combinations of tissues with cell types that are not usually undergoing morphogenetic movements. One especial defect of this approach that is not usually pointed out is that it is likely that cell surface properties will change during real morphogenetic movements and therefore that cell behaviour will also be changed. Artificial sorting out experiments tended to assume constant properties (at least following recovery from tissue disaggregation procedures) and that sorting would therefore go to completion based on initial differences in cells cohesiveness. Some of Steinberg's later experiments in this field (Wiseman *et al*, 1972) do point to modulations in cohesiveness, but do not really take this as far as a discussion of real morphogenetic movements.

Steinberg's DAH experiments used not only mixtures of disaggregated cells, but he also observed the spreading of coherent tissues on each others surfaces - and showed that the less cohesive type would spread to cover the more cohesive type.

Active cell motility is needed for cells to spread and sort out. It was found that cytochalasin B has an inhibitory effect on cell sorting and tissue spreading by means of inhibiting those involved cells which are actively moving (Maslow & Mayhew, 1972 and Steinberg & Wiseman, 1972).

Since none of the other mechanisms discussed above seem at all likely, differential adhesion is at least consistent with what is seen. But I have no direct evidence for cohesive differences between cortical and chromaffin cells.

In mouse, early embryonic stages showed chromaffin cells

intermingling with cortical cells, as individuals and patches at the periphery of the gland. A slow pattern of chromaffin cell accumulation near the centre occurred and eventually there formed a more or less centralised single mass, the medulla. The position of the medulla within the gland is variable, sometimes being very nearly central, sometimes being off to one side (see chapter 7 for details). This sorting out of adrenal tissues into medulla and cortex fits well with the predictions of the DAH.

Another piece of evidence for differential adhesion occurring during adrenal gland morphogenesis is that segregating cells possess microfilaments and microtubules, but not conspicuous locomotory processes. Some cells during, but not after segregation, and at the surface of patches, have flattened shapes. These characteristics of the segregating cells fit with sorting out essentially by surface adhesive differences, especially when we note that heterologous adhesions are smooth with relatively even intercellular spaces.

Two anomalies need explaining if differential adhesion is the mechanism of cell segregation in the adrenal:

a. Chick adrenal gland tissues are arranged in cords (corticals) and patches (chromaffin), and there is no complete segregation.

b. The failure of my experiment with halved mouse adrenals to show the re-internalisation of the medulla.

Both of the above anomalies could be explained by a change in surface adhesiveness or in cell motility after a certain stage in development. If the difference between surface/motility properties of cortical/medullary cells in chick is lost almost as soon as the chromaffin cells enter the cortical tissue mass, then

no further segregation would occur. Clearly, an alternative explanation would be differences in relative cell numbers. For example, in the chick, the proportion of chromaffin cells may be so low during cell segregation, that only a series of disconnected patches is formed, rather than a central medulla. I have not done cell counts at an early enough stage to test this possibility, but at the later stages I examined, the relative numbers of chromaffins to corticals seem too large for this explanation to hold, so at least by these stages, we must assume relative movements are no longer occurring.

Similarly in the mouse, we could postulate that once the cortical and chromaffin cells have segregated, cell surface differences are lost, so that exposure of chromaffin cells at the surface does not lead to their internalisation. This is not entirely convincing, since changes in the shape of the medullary-cortical boundary do continue after the stages when I did my adrenal halving experiments. Another explanation is simply that a better medium has to be found for maintaining the sensitive cells of the adrenal in culture. It might be worth trying the cutting experiment at earlier stages when cell segregation is still definitely occurring, but the result would be hard to interpret because of the incomplete segregation of tissues at the start of the experiment.

Morphometric analysis of adhesive differences in adrenal gland tissues is needed to gain more information about the way cortical and chromaffin cells are sorted out and arranged.

CHAPTER NINE

General Conclusions

Since each individual chapter has its own discussion on the work undertaken, it is the aim of this chapter to bring together general conclusions drawn from this study and to make recommendations for future investigation.

The aim of this study was to make a comparative study on the morphogenetic aspects of the development of the adrenal gland in mouse (embryos and postnatal) and chick (embryos and post-hatching).

The present work is the first detailed descriptive study to compare the morphogenesis of adrenal gland during both embryonic and postnatal life. My observations confirm that mammalian and avian adrenal glands are not structurally the same. They differ in their internal organisation though they have similar origins, the cortex from mesoderm and chromaffin tissue from neuro-ectoderm. The mouse adrenal gland shows a similar internal arrangement during early stages to that of the chick adrenal, but during later stages of development cells of the mouse adrenal sort out according to tissue type. This sorting out does not occur within the chick adrenal tissues: chromaffin cells remain as isolated patches amongst the cords of cortical cells.

The observations made regarding the development of the adrenal gland in mouse are as follows: the morphogenesis of the adrenal gland takes place during embryonic life and goes on for several weeks after birth, that is when the gland has assumed its adult characteristics. Both cortical and chromaffin tissues of

the adrenal are intermingled during early embryonic life and start to sort out into internal and external regions by the end of embryonic life.

The chromaffin tissue starts to join up in the centre of the gland about the 15-16th day of gestation and by the 17-18th day most chromaffin cells have reached the centre of the gland. By the end of 1st week of postnatal life, the chromaffin cells of the medulla have assumed their final position in the centre of the gland.

Cortical cells take the peripheral position on about the 16-18th day of gestation. At this time some cells show some features of the arrangement of the future zones of the adult cortex and by the end of the 1st week of postnatal life, the cortex possesses three zones (zona glomerulosa, fasciculata and the X-zone). The X-zone is a transitory zone which gets larger in the next few weeks and then start to degenerate later on. By the 5th week, the male adrenal cortex has three adult zones, zona glomerulosa, fasciculata and reticularis and the X-zone has already disappeared and as a result of its degeneration a capsule is formed between the cortex and medulla.

In the chick adrenal, cortical and chromaffin tissues are intermingled with each other during embryonic life and post-hatching life.

In both mouse and chick embryo adrenal glands, fibronectin was distributed randomly throughout the gland: under the capsule, between groups of cells of both cortical and chromaffin type and within blood vessels. It was also present within groups of cortical cells, but not within chromaffin groups.

Several methods of culturing adrenal tissues were employed. Unfortunately, it was hard to identify cell types in culture,

though several stains were used. Another problem with the cultures was the relatively rapid degeneration of tissues, which made interpretation of experiments of cell movements in culture rather difficult.

An unexpected result of my work was the discovery of cell death among the embryonic tissues in both mouse and chick adrenal glands, occurring in both cortical and chromaffin tissues.

Quantitative analysis of some features of mouse adrenal development showed that during embryonic life the cortical tissue grows rapidly whereas the chromaffin tissue grows more slowly. In postnatal life the growth of the cortical tissue is slow whereas chromaffin tissue appears to expand more rapidly.

This study leaves several questions for future investigation:

1. The role of dying cells with respect to adrenal gland development. What causes some cells of the adrenal gland to die at the time when the gland is forming and taking its final shape, and what is the morphogenetic role of this cell death. My suggestion that cell death allows space for cell sorting could be tested by a more detailed quantitative analysis of the distribution and frequency of cell death.
2. Relative movements of mouse adrenal tissues once cell sorting has occurred. This could be tested better if a culture medium could be found that kept adrenal tissue healthy over a longer period. 3. The scanning electron microscope internal appearance of the adrenal was particularly disappointing. Further work on different methods of fixation might reveal procedures that allowed observation of the shapes of cells during the

sorting process.

4. A morphometric study on the growth of the X-zone. Is X-zone growth the result of X-zone cell proliferation or the transformation of the inner zona fasciculata cells?

5. A study of the adhesive differences between cortical and chromaffin cells. This could be approached in two general ways: a) a morphometric analysis of cell junction frequency. I saw no obvious differences in time or location, but did not attempt a quantitative analysis. b) analysis of the presence of cell adhesion molecules, by means of specific antibody labelling.

6. A study of the earliest stages of chick adrenal development, to test the pattern of formation of the chromaffin cell patches, and the relative numbers of chromaffin and cortical cells at these stages.

My study seems to be the first to analyse the development of the adrenal in morphogenetic terms. It is inevitable, therefore, that this work has revealed several problems for further analysis. I believe that the adrenal offers considerable potential for the study of differential adhesion as a mechanism of morphogenesis in an organ where cell sorting actually occurs in development, as opposed to the artificial cell combinations which have mostly been studied in the past.

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