MORPHOLOGICAL AND FUNCTIONAL CORRELATIONS IN NORMAL AND NEOPLASTIC TISSUES

By

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FOREWORD

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"Morbid Anatomy" and "Histopathology" imply morphological studies and relate to the appearance of a disease process at a particular moment in time. However, disease is a dynamic process and the morphologist examining static appearances of a disease must interpret these features as part of a continuing sequence of biochemical and physiological abnormalities. In other words he must make a functional extrapolation from morphological aberrations. Such an extrapolation can only be made if the normal physiological and biochemical processes are understood, and the clinical sequelae of the abnormalities are known.

Descriptive pathology has led to a better understanding of disease and has enabled great advances to be made in the fields of clinical assessment, diagnosis and treatment. The giants of descriptive pathology have made their contribution to modern medicine and it is now becoming more and more important for the functional pathologists to stand on their shoulders in order to increase our understanding of disease. The functional interpretation of morphology is becoming less subjective and we are now at a stage when the clinician, the morphologist, the chemical pathologist and the experimentalist must pool their resources in order to make advances.

The theme of this thesis is "Functional Pathology",or function in relation to morphology. Two organs will be considered: the human adrenal cortex, and the testis. The function of both of these in health and disease can be

assessed readily and alterations may be reflected in morphological changes.

The adrenal cortex produces steroid hormones and the production rate of these increases in times of stress. The well known morphological changes of hyperplasia and lipid depletion of the adrenal cortex occur in association with increased steroid output (Symington, 1969). However, in primary adrenocortical pathological states, morphological changes do not always accurately reflect functional aberrations. Hyperactivity of the adrenal cortex due to hyperplasia or neoplasia of steroid-producing cells would be expected to produce the clinical effects of Cushing's syndrome, and in many cases this relationship is true. However, hyperactivity of the inner cortical zones may occur in the absence of clinical symptoms, or may produce Conn's syndrome or the adrenogenital syndrome. The explanation of this lies in the fact that individual cells are capable of producing different classes of steroid hormones notably glucocorticoids, sex steroids, and mineralocorticoids, as well as many biologically inactive steroids. The clinical syndrome produced depends on the amount of steroid produced, and the relative proportion of the different classes of steroids. Therefore, the hormonal effects of adrenocortical hyperplasia or neoplasia cannot be surmised on morphological features alone, and a single morphological pattern may be associated with many different syndromes.

The interstitial tissue of the testis is another steroid- producing endocrine gland secreting androgenic (virilising) steroids including testosterone.

Therefore a functioning tumour of testicular interstitial cells would be expected to cause virilisation. This often happens, especially noticeable in prepubertal subjects, but some interstitial cell tumours of similar morphology may result in feminisation (Neville, 1976; Symington & Cameron, 1976).

It is clear, therefore that although functional interpretation of morphological changes is often valid, the two are not always related and the pathologist's assessment must always be guarded. In the field of tumour pathology, many, if not all, tumours produce substances which are abnormal qualitatively, quantitatively, or temporally. These substances, usually hormones, enzymes or antigens, may be secreted appropriately or inappropriately. The functional tumour pathologist must be aware of the secretory activity of the tumour he is examining, and if possible should try to ascertain which morphological features are associated with which particular secretory products, and should determine if a consistent relationship exists.

In my studies I have attempted to correlate function with morphology considering both normal and neoplastic tissue. The normal developing foetal testis undergoes a series of morphological changes and this is related temporally and spatially to the developing genital ducts and tracts. I have tried to correlate steroidogenic function with these changes. I have also examined neoplastic adrenal and testicular tissue with special reference to their function - or lack of overt function - and the associated clinical effects.

I hope to illustrate the concept that functional differentiation of cells can occur irrespective of histological

appearances. As examples I shall describe the function of foetal testicular cells in the developing rat foetus in utero, and also in tissue culture. I shall also present data correlating the production of alpha-fetoprotein with specific cell types in testicular tumours, and I shall describe the functional capacity of certain adrenocortical carcinomas.

SECTION I : EXPERIMENTAL STUDIES

CHAPTER 1

INTRODUCTION

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INTRODUCTION

FUNCTIONAL ASPECTS OF THE FOETAL TESTIS

1. Embryological development of the male urogenital system

The genetic sex of mammals is determined at conception, the heterozygous (XY) state being the male genotype. The early embryo first passes through a non-sexual phase before the genital apparatus appears. This is followed by an indifferent sexual phase when gonadal tissue appears in both sexes but shows no ovarian or testicular differentiation, and there is no phenotypic difference between male and female genotypes. Testicular differentiation of the indifferent gonad is determined by the Y chromosome probably interacting with the X chromosome. Federman (1973) states that structural gene information for testicular differentiation is carried on the X chromosome but this is under the regulating control of genes in the Y chromosomes. Thereafter, the foetal testis stimulates male phenotypic development. In the female genotype (XX) ovarian differentiation does not occur until after sexual differentiation of the genital ducts, and the foetal ovary apparently plays no part in foetal sexual development. Female development of the genital ducts occurs in the absence of testes, that is, if the ovaries are present, or if no gonad is present.

The internal and external genitalia are derived from the genital tracts and the urogenital sinus (Fig. 1.1). The chronological events in sexual differentiation are represented

Fig. 1.1. Development of the internal genitalia in the human embryo.

(a) Sexually undifferentiated stage. An indifferent gonad is present in both sexes medial to the mesonephros (primitive kidney), and paired genital tracts extend from the urogenital ridge down the dorsolateral aspect of the coelomic cavity to the urogenital sinus. Each genital tract contains 2 genital ducts: the Wolffian duct derived from the mesonephric duct, and the Mullerian duct which originates de novo.

(b) Male differentiation is first evidenced by the indifferent gonad developing into a recognisable testis. Testicular secretions cause degeneration of the Mullerian duct and stabilisation of the Wolffian duct. Under the action of testicular androgens, the Wolffian duct develops into the epididymis, vas deferens and seminal vesicle; and the urogenital sinus develops into the prostate.

(c) Female differentiation does not require a functioning embryonic gonad. The gonad remains indifferent, the Wolffian duct regresses and the Mullerian duct, which is not under any inhibitory influence, develops into the fallopian tube, uterus and upper vagina, the urogenital sinus forming the vagina below the hymen.

(after Jost, 1971)

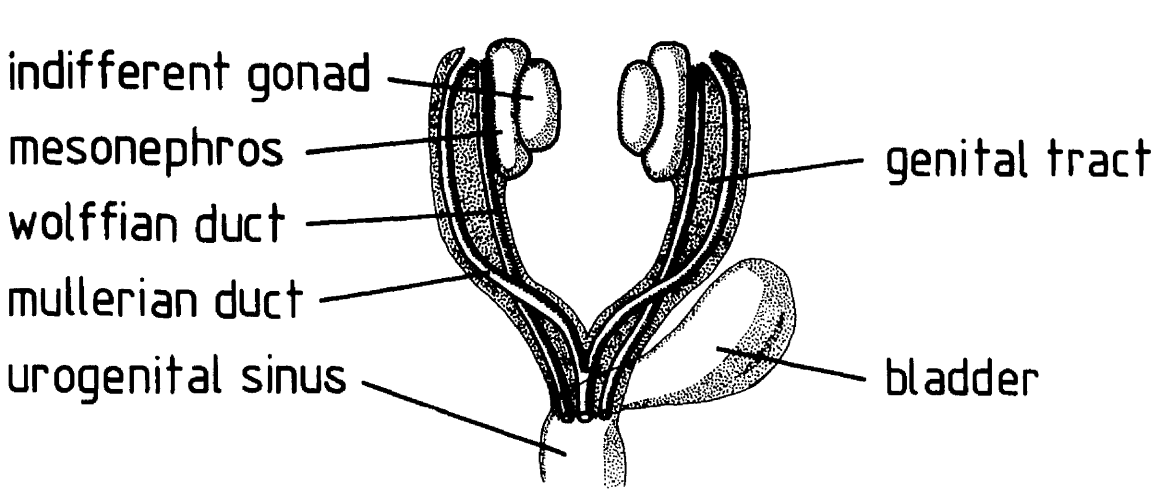


Fig. 1.1.a

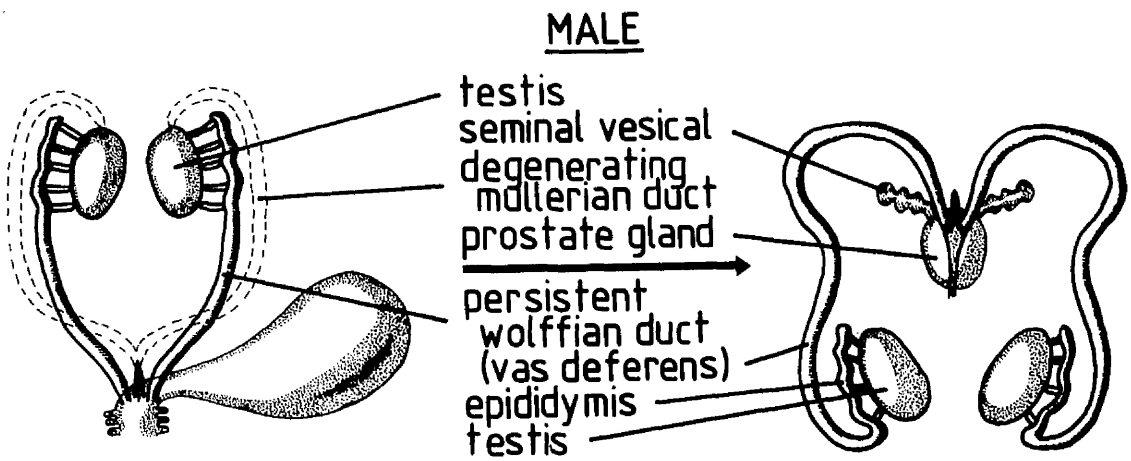


Fig. 1.1.b

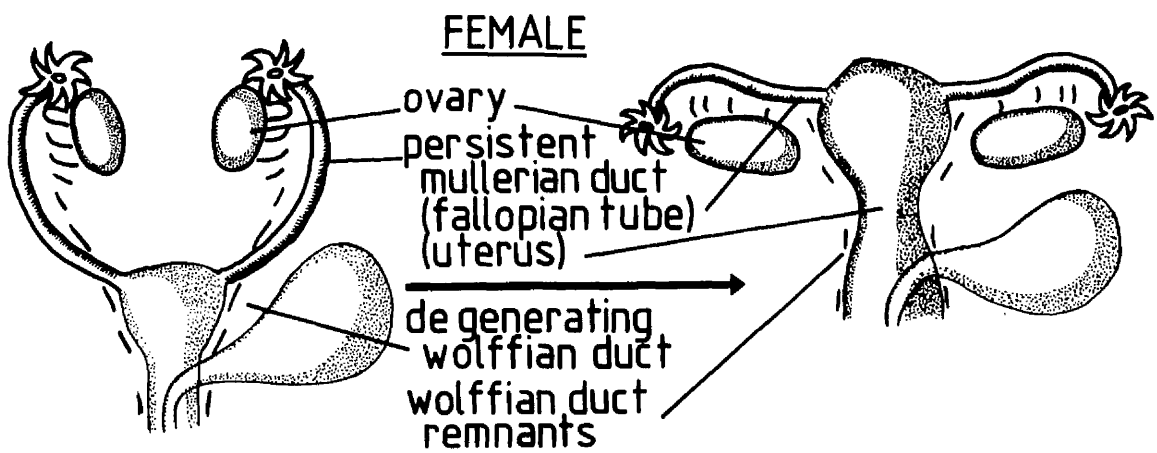


Fig. 1.1.c

in Fig. 1.2. The Wolffian duct is primarily an excretory duct (pronephric and mesonephric duct) but in the mammal, it does not retain any excretory function and becomes incorporated into the developing genital system. The Mullerian duct, however, is primarily a genital duct and has no excretory function. It appears firstly as a groove in the lateral aspect of the cranial end of the mesonephric ridge lateral to the pre-existing Wolffian duct. The groove deepens, becomes tubular(remaining open at the cranial end) and extends towards the urogenital sinus. It crosses over to the medial aspect of the Wolffian duct just before it reaches the urogenital sinus. In the male, the Mullerian duct regresses and persists only as the appendix of the testis and the prostatic utricle. Regression commences anteriorly before the caudal end meets the urogenital sinus, therefore a wave of development and regression of the Mullerian duct passes along the genital tract with both processes occurring simultaneously.

2. Endocrine activity of the foetal testis

The role of the testis in the development and regression of the genital ducts has been examined extensively since Bouin and Ancel (1903 a&b) first suggested that the foetal testis secreted hormones which controlled foetal sexual development. In 1947, Jost described a method for castrating foetal rabbits *in utero* without terminating pregnancy. He demonstrated that the foetal testis is required for stabilisation and further development of the Wolffian duct; for the differentiation of the urogenital sinus into the prostate gland and external genitalia; and also for the regression of the Mullerian duct (Jost, 1947b).

Fig. 1.2. Chronological events in sexual differentiation of the rat embryo

The Wolffian develops at 9½ days gestation as the pronephric duct, and later the mesonephric duct. The indifferent gonad appears at 11½ days. The first sign of sexual differentiation occurs at 14½ days when, in the male, the testis enlarges and becomes histologically differentiated, as evidenced by the presence of primitive seminiferous tubules and interstitial cells. The Mullerian duct commences its development at its anterior end at 13½ days, and then progresses posteriorly meeting the urogenital sinus at 16½ days. In the male, Mullerian duct regression commences anteriorly at 14½ days; that is, before posterior development has been completed. However, in the female, this duct persists and develops into female genitalia. At 17½ days, the Wolffian duct is stabilised in the male and develops into male genitalia, but in the female, the Wolffian duct regresses because of lack of stabilising androgens.

(Data from Jost, 1967; Price & Pannabecker, 1959; Morishge et al., 1973; Torrey, 1943, 1945)

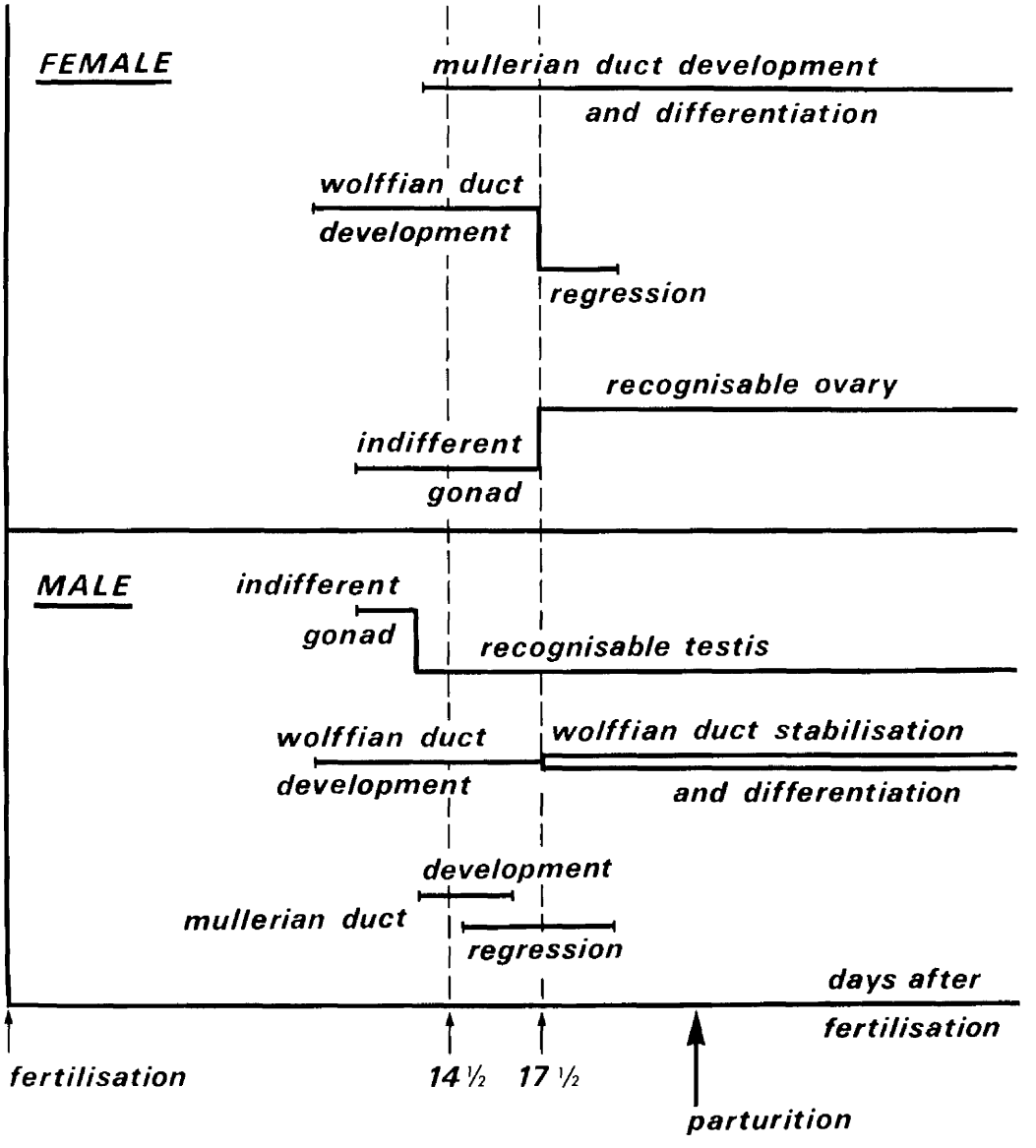

FEMALE
mullerian duct development
and differentiation
wolffian duct
development
regression
recognisable ovary
indifferent
gonad
MALE
indifferent
gonad
recognisable testis
wolffian duct
development
wolffian duct stabilisation
and differentiation
development
mullerian duct
regression
days after
fertilisation
fertilisation
14 ½
17 ½
parturition

Fig. 1.2

A single testis is sufficient requirement for the development of the prostate and external genitalia. However, unilateral castration demonstrates that each testis only acts on its adjacent genital ducts and not the contralateral ducts. Stabilisation of the Wolffian ducts in the castrated male foetus (Jost, 1947c) or in the intact female foetus (Jost, 1947a&b) can be achieved by exogenous androgenic steroids, but these fail to cause regression of the Mullerian ducts.

Reviewing experiments on foetal testicular endocrinology, Jost (1953) postulated that the foetal testis secretes at least 2 hormones: firstly, an androgenic steroid which stabilises the Wolffian ducts and stimulates the development of the Wolffian ducts and urogenital sinus into internal and external genitalia; and secondly, a non-androgenic Mullerian inhibitory factor (MIF) whose chemical nature is unknown. Testosterone* is the main androgenic steroid produced by the testis, and this steroid is probably responsible for Wolffian duct stabilisation, whereas its 5α-reduced metabolite (dihydrotestosterone) is probably responsible for male differentiation of the urogenital sinus (Schultz & Wilson, 1974). Androgens not only fail to inhibit the Mullerian duct, but in the opossum large doses of testosterone propionate cause enlargement of the Mullerian derivatives (Burns, 1945). Jost (1953) showed that deoxycorticosterone acetate (DOCA) prepared in a solution containing 5% benzyl alcohol causes Mullerian duct regression <u>in vitro</u> in foetal rabbits.

* Trivial names or abbreviated names of steroids and steroidogenic enzymes are used in the text. A full list of abbreviated names, trivial names and systematic names are given in Appendices I & II.

Pure DOCA is devoid of Mullerian inhibitory activity but 5% benzyl alcohol alone is active. This is probably due to non-specific toxicity.

Many substances have been examined for Mullerian inhibitory activity (Table 1.I) but none has been shown to have the same specific effect as natural MIF.

3. In vitro assay system for Mullerian inhibitory factor (MIF)

An in vitro organ culture technique has been developed by Picon (1969) for the study of Mullerian inhibition. Rat foetal genital tracts are explanted from 14½ days post coitus embryos and maintained in organ culture for 3 days. Normal development of the genital ducts takes place in culture. At 14½ days the Mullerian ducts have formed anteriorly and are sensitive to inhibition by testicular MIF (Fig. 1.2). After 3 days in culture, equivalent to 17½ gestation, Mullerian inhibition is evident if MIF is present, and the Wolffian ducts remain intact in both sexes or show only early anterior regression. The state of the genital ducts is determined histologically. These experiments show that the procedure involved in explanting foetal genital tracts do not interfere with subsequent development and differentiation of the genital ducts if the tracts are cultured in a suitable environment.

Using this system Picon and Josso (vide infra) have studied facets of the Mullerian inhibitory factor. The Mullerian ducts are inhibited unilaterally by the presence of autologous (Picon, 1969) or heterologous (Picon, 1970) foetal rat testes. There is interspecific activity of MIF, the foetal testes of rabbit (Picon, 1971), human (Josso, 1970b,1971b) or ox (Josso, 1973) being able to inhibit the Mullerian ducts of rat. MIF is produced by the foetal rat testis from 13½ days

Table 1.I. Effect of various substances on the genital ducts of mammals.

Substance *	Effect on the		Reference
	Wolffian duct	Mullerian duct	
testosterone	stabilisation	no regression	Jost, 1947(c), 1971(a)
17 methyl testosterone	stabilisation	no regression	Jost, 1947(b)
9α-fluoro-11β-hydroxy-17-methyl testosterone	stabilisation	no regression	
3β-androstanediol	not stated	no regression	Josso, 1971(a)
3α-androstanediol	not stated	no regression	Josso, 1971(a)
androstenediol	stabilisation	no regression	Josso, 1970(a)
5α-dihydrotestosterone	stabilisation	no regression	Schultz and Wilson, 1974; Josso, 1971(a)
5β-dihydrotestosterone	inactive	no regression	Josso, 1971(a)
dehydroepiandrosterone (DHA)	partial stabilisation	no regression	Josso, 1970(a)
DHA sulphate (DHAS)	no effect	no regression	Josso, 1970(a)
17 ethinyl testosterone	not stated	no regression	Jost, 1947(b)
oestradiol	slight stabilisation	maintenance	Jost, 1971/72 Forsberg et al., 1968
deoxycorticosterone acetate (DOCA)	no effect	no regression	Jost, 1953
prostaglandins E, E_2, $F_{2\alpha}$	no effect	no regression	Jost, 1973
prostaglandins A_2, B_1	not stated	reduced diameter but not same as natural MIF	Josso, 1974(a)
prostaglandin E_1	not stated	no regression	Josso, 1974(a)
olive oil / benzyl alcohol	not stated	regression (toxic)	Jost, 1953
cyproterone acetate	prevents differentiation	no regression	Forsberg et al., 1968; Jost, 1967, 1971/72
cyanoketone	does not inhibit stabilisation	not stated	Josso, 1970(c); Jost, 1971/72
cyclic AMP	not stated	prevents regression	Picon, 1976(b)

* Systematic nomenclature of steroids is given in Appendix I

post coitus to 13 days post partum (Picon, 1970); by the human foetal testis from 7 to 24 weeks gestation (Josso, 1970b); and by the foetal calf testis (Josso, 1973).

In the human foetus, MIF production after 24 weeks gestation falls off towards term, and is not produced by the post partum, prepubertal, or pubertal testis (Josso, 1972b). Foetal calf testis does not produce MIF in late pregnancy (Josso, 1973).

The Mullerian ducts are not inhibited by testosterone, dihydrotesterone, 3α- or 3β-androstanediol, or prostaglandins E_1, A_2, or B_1 in organ culture (Josso, 1971b, 1974a), nor are they inhibited by the human foetal adrenal or ovary (Josso, 1971b). Human MIF is permeable through the vitelline membrane of a hen's egg, but not through Visking cellulose, a membrane which is permeable to testosterone but impermeable to molecules greater than 15,000 Daltons (Josso, 1972a). This indicates that MIF is not a steroid in the free form. MIF, but not testosterone, can be concentrated by dialysis and ultracentrifugation (Josso, 1974d); and it can be partially purified by gel filtration on sephadex G200 or by column chromatography on Bio-gel A-5m. These data indicate a molecular weight of between 200,000 and 295,000 Daltons (Picard & Josso, 1976).

MIF is produced in tissue culture by whole foetal calf testes, or isolated foetal calf Sertoli cells, but not by isolated foetal calf Leydig cells (Josso, 1973). Human foetal testes made devoid of germ cells by exposure to irradiation in vitro produce MIF in culture (Josso, 1974b). Monolayer culture of isolated, foetal calf Sertoli cells, but not isolated foetal calf Leydig cells, produce MIF (Josso, 1974c).

These experiments indicate that Sertoli cells produce MIF.

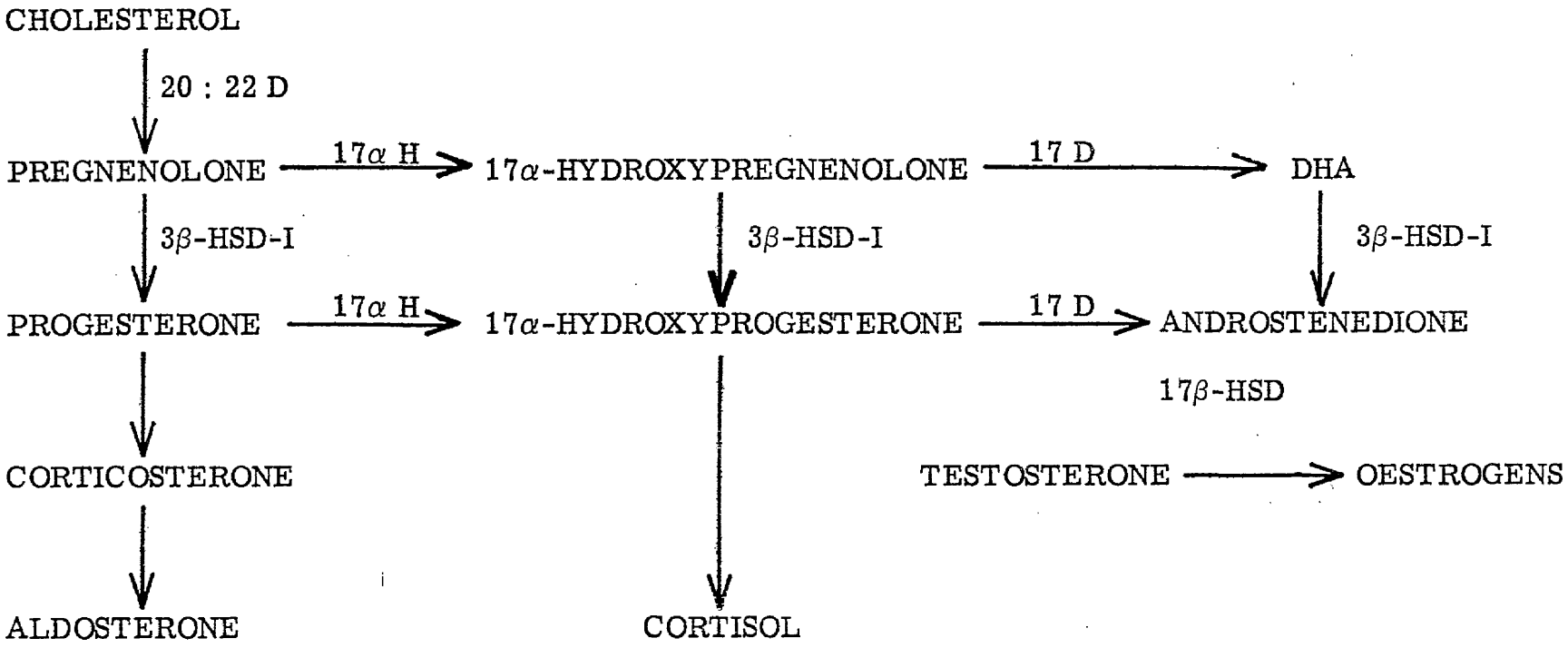
MIF is present in the protein rich fraction of dialysed culture medium (Josso, 1974d) and is destroyed by heating (Josso, 1974d; Josso et al., 1975) or by blockade of sulphydryl groups by 0.5M iodoacetic acid (Josso et al., 1975). It is not produced in culture if protein synthesis is inhibited by cycloheximide (Josso et al., 1975). MIF activity is stable to 0.01 M dithioerythritol, a reducing agent, but is destroyed by 8M urea or by 0.1% SDS depolymerising agents (Picard & Josso, 1976).

4. Possible role of progesterone in Mullerian duct inhibition

The above experiments clearly indicate that MIF is not an androgenic steroid; is not a steroid in free form; and is probably a protein or protein complex whose production requires Sertoli cells. It is possible, however, that MIF may be a steroid combined to a steroid binding protein, and that this steroid may be progesterone. The possibility that progesterone may be involved in the Mullerian inhibition has not been previously postulated, and the effects of progesterone on the Mullerian ducts in culture have not been published.
The following points support the hypothesis that progesterone may be part of the Mullerian inhibitory factor:-

a) The foetal testis, having the enzymes necessary for synthesis of testosterone (Acevedo, et al.,1963; Lipsett & Tullner, 1965; Picon, 1976; Rice et al., 1966; Warren et al., 1972), has the capability of producing progesterone (Fig.1.3).

b) The enzyme system 3β-hydroxysteroid dehydrogenase-



20 : 22 D	C 20 : 22 desmolase*
17α H	17α-hydroxylase
3β-HSD-I	Δ^5-3β-hydroxysteroid dehydrogenase - isomerase systems
17 D	17 desmolase
17β-HSD	17β-hydroxysteroid dehydrogenase

* Recommended systematic nomenclature of steroids and enzymes is given in Appendices I and II.

Fig. 1.3. Main steroidogenic pathways (schematic) *

isomerase (3β-HSD-I), required for conversion of pregnenolone to progesterone (Fig. 1.3) is present in the very early stages of testicular development, before the time when the testis exerts its inhibitory action on the Mullerian duct Baillie, 1965; Baillie & Griffiths, 1964; Baillie et al., 1966ab&c; Bloch et al., 1971; Haffen, 1969; Hart, et al., 1966; Jirasek, 1970; Lipsett & Tullner, 1965; Moon & Raeside, 1972; Picon, 1976a).

c) At the start of Mullerian duct inhibition, the 3β-HSD-I acts preferentially on pregnenolone as substrate. Preference for 17α-hydr oxypregnenolone and DHA is not attained until later in embryonic life when androgens are required for Wolffian duct stabilisation (Baillie, 1965; Baillie & Griffiths, 1964; Baillie et al., 1966b&c).

d) The foetal testis produces progesterone, but not testosterone, at the time of Mullerian duct inhibition (Bloch et al., 1971b; Haffen, 1969; Jirasek, 1970; Siiteri & Wilson, 1974; Warren et al., 1973; Wilson and Siiteri, 1973).

e) After Mullerian duct inhibition, when stabilisation of the Wolffian duct occurs, the secretion of MIF by the foetal testis falls progressively as the output of testosterone rises (Baillie & Griffiths, 1964; Josso, 1970b; Lipsett & Tullner, 1965; Noumura et al., 1966; Pointis & Mahoudean, 1977; Picon, 1970,1976a; Siiteri & Wilson, 1974; Warren et al.,1973; Wilson & Siiteri, 1973). If this is due to conversion of MIF to testosterone, then progesterone is a likely candidate for MIF.

f) Progesterone has an inhibitory effect on the prepubertal and mature endometrium, a Mullerian duct derivative, causing

reduction of cell division (Martin et al., 1973; Trams et al., 1973a&b).

g) Premature and mature endometrium and myometrium contain specific progesterone receptors (McGuire et al., 1974; Philibert & Raynaud, 1973,1974; Trams et al., 1973b; Warembourg, 1974).

h) Oestrogens can stabilise the Mullerian duct (Forsberg et al., 1968; Jean, 1966) and therefore have an anti-MIF action. Oestrogens and progesterone have known antagonistic activities (Lerner et al., 1966; Trams et al., 1973a&b).

j) Dibutyryl cyclic AMP prevents the inhibitory effect of testicular MIF in vitro (Picon, 1976b). Cyclic AMP is known to stimulate steroidogenesis and may act by causing metabolism of progesterone to another steroid, for example testosterone.

It is difficult to examine the effects of progesterone on the Mullerian ducts in vivo. To achieve this, two different experiments are required: the first preventing endogenous progesterone reaching the Mullerian ducts; and the second subjecting them to excess exogenous progesterone.

All mammals require progesterone for the maintenance of pregnancy (Davies & Ryan, 1972). If the Mullerian ducts are to be deprived of endogenous progesterone stimulation, complete inhibition of progesterone synthesis must be achieved and this results in abortion of the developing foetuses. Therefore, examination of further foetal sexual development in a progesterone-free environment in vivo is not possible.

Cyanoketone (2α-cyano-4,4,17α-trimethylandrost-5-ene-17β-ol-3-one) is an inhibitor of 3β-hydroxysteroid dehydrogenase

and steroid Δ-isomerase (Goldman, 1971/72; Goldman et al., 1966; Neville & Engel, 1968a&b). In the mouse, small doses of cyanoketone given to pregnant mothers have no effect on sexual development of the foetuses and do not prevent Mullerian duct regression in the male; and large doses result in foetal resorption (Bloch et al., 1971a). Cyanoketone has been shown to be a more effective enzyme inhibitor in the adrenal cortex than in the testis in vivo (Di Prisco et al., 1971/72); and cyanoketone given to pregnant rats fail to block completely 3β-HSD-I in the foetal testes (Bloch, et al., 1971b). Therefore, when Mullerian ducts regress normally in male foetuses of pregnant rats treated with cyanoketone, it cannot be concluded that regressed Mullerian ducts were not exposed to progesterone.

Foetal testicular MIF has a very localised effect and only influences the adjacent genital tract. This suggests that MIF is not a systemically acting humoral agent, but passes directly from the testis to the ipsilateral Mullerian duct. MIF injected into the pregnant mother may not cross the placental barrier, or may be metabolised by maternal tissue, placental tissue or foetal tissue. Any residual MIF in the foetal circulation will then act as a systemic hormone and will therefore most likely be devoid of Mullerian inhibitory activity. This means that if progesterone has Mullerian inhibitory activity, such an action will not be demonstrated by injecting the pregnant mothers with excess quantities of progesterone.

Progestogens have been given to pregnant humans and experimental animals, and these substances do not cause Mullerian inhibition in female foetuses. In some cases they

produce a certain degree of masculinisation of the urogenital sinus (Foote et al., 1968; Jones & Wilkins, 1960; Revesz et al., 1960; Suchowsky & Junkman, 1961; Suchowsky et al., 1967; Wilkins, 1960; Wilkins et al., 1958), and synthetic progestogens are more virilising than progesterone itself. This androgenicity of progesterone on urogenital derivatives has been known for a long time (Clausen, 1942; Green et al., 1940). Progesterone and progestogens are virilising compounds and Mullerian inhibition is a virilising effect of the foetal testis. This is further evidence that a Mullerian inhibitory effect of progesterone should be considered and examined.

In this thesis an attempt has been made to determine if progesterone is the Mullerian inhibitory factor. This has been tackled in two ways. Firstly, progesterone was added to foetal rat genital tracts in organ culture to see if this could cause Mullerian inhibition in vitro. Secondly, foetal rat testes were maintained in monolayer tissue culture, and the endogenously produced steroids examined by radioimmunoassay and high pressure liquid chromatography. The results are present in chapters 3 and 4. The organ culture studies follow the methods of Picon (1969) and Josso (1970a) already described (vide supra). The monolayer culture studies are new and will be described in detail.

5. Foetal testicular steroidogenesis

The foetal testis is a steroid-producing organ and there are many direct and indirect methods of demonstrating steroid synthesis, in particular androgen production (Zaaijer, 1975), viz:-

a) The foetal testis has an androgenic (virilising) effect on foetal target organs, and this effect has been demonstrated *in vitro* and *in vivo*.

b) Target organs have androgenic receptors, and these receptors develop roughly about the same time as the testis begins to produce steroids; and just before morphological changes occur in the target organs as a result of androgenic stimulation.

c) The level of androgens in the foetal serum has been measured, and extracts of foetal testes have been shown to contain steroids. The nature and quantity of these steroids have been examined.

d) Steroidogenic enzymes have been demonstrated in foetal testes by histochemical techniques and these enzymes are required in the biosynthesis of testosterone.

e) The presence of testicular interstitial cells, which are known to be associated with androgen production, can be detected histologically at a certain stage of development, and electron microscopy has shown that these cells have the ultrastructural characteristics of steroid-producing cells.

f) The synthesis of androgens from added precursors has been demonstrated in incubations of foetal testicular slices and homogenates.

g) Endogenous production of androgens by foetal testes in tissue culture, either organ culture or monolayer culture, has been demonstrated.

An attempt has been made in this thesis to examine the stage at which foetal testicular steroidogenesis is first evident, and to study the changing profile of steroid output at various gestational ages in the rat foetus. The various methods for examining these facets of foetal testicular steroidogenesis as outlined by Zaaijer are not completely satisfactory for the following reasons:-

a) Jost (1953, vide supra) examined the androgenic effect of the foetal testis on sexual differentiation and clearly demonstrated that the foetal testis produces androgens. However, the sex ducts (Wolffian ducts) are not responsive to androgens until a certain stage of embryonic development. It follows, therefore, that the time of morphological changes in the sex ducts does not necessarily indicate the precise time when androgenic secretion commences: this is because any androgenic secretion occurring before the sex ducts are responsive will not be associated with morphological changes.

b) Androgens can only have their effect on target organs after such organs have developed androgen receptors (Zaaijer, 1975), but the time of appearance of receptors need not necessarily coincide exactly with the time when androgen secretion commences. Similarly, the time of appearance of gonadotrophin receptors on foetal testicular cells can be measured, but this too is not an absolute indication of steroidogenic activity. George et al., (1978) demonstrated HCG receptors in foetal

rabbit testes at 18 days gestation, but such receptors are apparently not functional till 20 days gestation.

It is known that foetal testicular steroidogenesis is stimulated by HCG (Abramovich et al., 1974; Ahluwalia et al., 1974; Huhtaniemi et al., 1977a&b; Weniger et al., 1967; Weniger & Zeis, 1975) and by LH (Weniger et al.,1967; Weniger & Zeis, 1975). However, steroidogenesis can occur in the absence of trophic hormone (Picon, 1976a), and the exact role of chorionic gonadotrophin and of foetal pituitary gonadotrophin on the development of the foetal testis and its subsequent steroidogenic activity is not yet clear. George et al., (1978) have demonstrated that the development of 3β-HSD-I activity and the morphological appearance of interstitial cells can occur in vitro in the absence of trophic stimulation.

c) Steroids can be extracted and identified from foetal testes,indicating in vivo steroid production. Veyssiere et al. (1976) extracted testosterone from foetal rabbit testes. Reyes et al.(1973) extracted testosterone from human foetal testes and demonstrated the presence of this steroid at 10 weeks gestation with peak levels at 11-14 weeks. These authors (Reyes et al., 1974) also demonstrated corresponding levels of testosterone in human foetal serum. Huhtaniemi et al.(1970) extracted steroids from human foetal testes and showed that testes from 12-24 weeks gestation foetuses contained mainly pregnenolone and testosterone. They claim to have been the first group to prove that human foetal testes synthesise testosterone in vivo, but this method cannot exclude the possibility that the steroids may have been produced elsewhere

and taken up by the testes. These authors tried but failed to identify progesterone in these relatively mature foetal testes.

d) A good indication of the steroidogenic capacity of the foetaltestis is given by the demonstration of steroidogenic enzymes within foetal testicular cells. Such enzymes can be demonstrated histochemically by staining techniques or biochemically using tissue homogenates. The steroidogenic enzyme studied most extensively by histochemistry is 3β-HSD-I which is present in the mouse testis at 11 days gestation, the stage at which the mouse foetal gonad is first recognisable as a testis (Hart et al., 1966). This enzyme acts preferentially on different substrates at different gestational ages. It acts on pregnenolone in the early foetal testis, and on DHA later in foetal life, but it does not act preferentially on 17-hydroxy pregnenolone till after parturition (Baillie, 1965; Baillie & Griffiths, 1964; Baillie et al., 1966ab&c). Biochemical demonstration of 3β-HSD-I activity in foetal rat testes has been described by Picon (1967). It must be stressed that the demonstration of steroidogenic enzyme activity in tissues does not necessarily imply that the tissue is actively secreting steroids in vivo. Moreover, identification of the various enzymes present does not give a true indication of the profile of steroids produced and released.

e) The interstitial (Leydig) cells are the main steroidogenic cells of the testis; however, demonstration of steroidogenic type structures on electron microscopy does not necessarily imply that steroidogenic activity has started. In fact, steroidogenic enzymes are present in the foetal testis before Leydig cells are recognizable (Baillie, 1965; Baillie & Griffiths,

1964 ; Baillie et al., 1966a&b) and these enzymes are even demonstrable in the genital ridge before germ cell migration (Baillie et al., 1966a).

f) Many workers have demonstrated the steroidogenic capacity of foetal testes by showing that labelled precursors are converted into steroid hormones in vitro. These precursors are incubated with tissue slices or homogenates in metabolic flasks, and the metabolic products are extracted and identified at the end of the incubation period. Incubations of human foetal testes have demonstrated testosterone synthesis from labelled acetate, cholesterol, pregnenolone, pregnenolone sulphate or progesterone (Acevedo et al., 1963; Ahluwalia et al., 1974; Bloch, 1966; Bloch et al., 1962; Huhtaniemi et al., 1977a; Payne et al., 1975; Siiteri & Wilson, 1974). Similar results have been obtained with incubations of foetal testes from mouse (Bloch et al., 1971a; Weniger et al., 1967; Weniger & Zeis, 1972,1975), rat (Bloch et al., 1971b; Noumura et al., 1966; Warren et al.,1972,1973) monkey (Huhtaniemi et al., 1977b), rabbit (Bloch et al., 1971b; Lipsett & Tullner, 1965; Wilson & Siiteri, 1973), and horse (MacArthur et al., 1967). In these incubations, steroidogenic activity is stimulated by HCG (Ahluwalia et al., 1974; Huhtaniemi et al., 1977a&b; Weniger & Zeis, 1975), LH (Weniger & Zeis, 1975) or cyclic AMP (Ahluwalia et al.,1974).

Incubations of foetal testes demonstrate the pattern of steroid synthesis, and this pattern is seen to be dependent on gestational age. Early foetal testes produce progesterone in greater quantities than testosterone, whereas foetal testes later in gestation produce testosterone and androstenedione as

the main steroid secretory products, and very little progesterone is detected (Ahluwalia et al., 1974; Bloch, 1966; Bloch et al., 1962; Bloch & Benirschke, 1965; Lipsett & Tullner, 1965; MacArthur et al., 1967; Noumura et al., 1966; Siiteri & Wilson, 1974; Warren et al., 1972,1973; Weniger & Zeis, 1972, 1975; Wilson & Siiteri, 1973).

Examination of steroid synthesis from added precursors in short term incubations has the disadvantage that the tissue under examination may not be in a steady state because of the trauma and disruption caused by preparing the tissue slices or homogenates. Also, the effects of endogenous precursors stored within the cells or the effect of trophic hormone attached to the tissue cannot be assessed. Many of the unknown variables of short term incubations can be avoided by examining the steroidogenic synthesis from basic molecules using long term tissue cultures of foetal testes, either in organ culture or monolayer culture.

g) Ortiz et al.(1966) maintained foetal guinea pig testes in organ culture. These authors demonstrated that androgenic substances were secreted by the testes in culture and these androgens stimulated the growth of rat ventral prostate glands placed adjacent to the foetal testes in the same organ culture dish. However, this is a bioassay system with an imprecise histological end-point which is dependent on morphological changes in the prostatic acini. This end-point is not quantitative, and also it lacks specificity because substances such as progesterone are also known to stimulate the prostate (vide supra). Picon (1967) kept rat foetal testes in long term organ culture and she was able to detect 3β-HSD-I activity in the cultured tissue by biochemical methods.

Weniger and co-workers showed that foetal mouse testes in organ culture for 24 hours could convert acetate or progesterone to testosterone (Weniger et al., 1967; Weniger & Zeis, 1972); and also that similar cultures were stimulated in vitro by LH and HCG to produce increased amounts of testosterone and androstenedione from added acetate (Weniger & Zeis, 1975). Rice et al. (1966) showed that foetal human testes at 6½ months gestation produced pregnenolone, progesterone and DHA from acetate after 9 days in culture: smaller amounts of testosterone and androstenedione were also found. Abramovich et al. (1974) also using organ culture techniques demonstrated testosterone output by human foetal testes maintained in culture for one day: this was endogenous testosterone production without added precursors, and testosterone synthesis was stimulated by HCG.

Picon (1976a) prepared and maintained foetal rat testes in organ culture for 3 days. Cultures prepared from 13½ days gestation foetuses showed no detectable testosterone production in the first day of culture, but subsequently testosterone output from these cultures developed and increased in vitro. Cultures prepared from 14½ days gestation foetuses showed slight initial testosterone output, and this increased over the 3 days in culture. However, cultures prepared from older foetuses showed maximum testosterone output in day 1 of culture, and this diminished in subsequent days in vitro. Maximum day 1 output was observed in cultures prepared from 17½-18½ days foetuses. Brinkman (1975) found similar results with organ cultures of foetal guinea pig testes, and demonstrated that in this species, foetal testicular testosterone synthesis was

demonstrable at 25 days gestation, 4 days before differentiation of the Wolffian duct.

George et al. (1978) examined organ cultures of foetal rabbit testes and demonstrated that early foetal testes devoid of recognizable interstitial cells, and without evidence of 3β-HSD-I activity, could develop both of these characteristics in culture in the absence of trophic hormones. The 3β-HSD-I activity developed at 18 days gestation at which time testicular cells developed HCG receptors. However, testosterone synthesis was not apparent till 20 days gestation.

6. Monolayer culture of foetal testes

In our laboratory, monolayer culture is preferred to organ culture because there is less chance of artifact arising due to diffusion of nutrients or products through the lumps of tissue. Also, the immediate cellular environment can be controlled more accurately, and viable individual cells can be observed readily by inverted phase-contrast microscopy. It has been shown that adrenal cells in monolayer culture retain many of their in vivo functions, but these functions are influenced by cell density in culture and also by the presence or absence of trophic hormone in the culture medium (O'Hare & Neville, 1973a, b&c; O'Hare et al., 1978). Having gained experience with adrenocortical cells in culture, the function of testicular cells in culture was then examined. As a marker of steroidogenic activity, a radioimmunoassay for testosterone was developed. However, detection of testosterone secretion gave no indication of the synthesis and release of steroid precursors, in particular progesterone.

Therefore, I developed a radioimmunoassay for progesterone in order to detect if this steroid - a precursor of testosterone - was released by the cells in culture. In addition, culture media were examined by high pressure liquid chromatography (HPLC) in order to examine the complete profile of steroids released.

Rat adult interstitial cells of the testis in monolayer culture initially produce testosterone, but after a few days in culture, testosterone secretion diminishes and progesterone secretion increases (Khatim & O'Hare, 1976), a situation analogous to the "spontaneous luteinisation" of ovarian granulosa cells in culture (O'Hare et al., 1978). Interstitial cells from adult and prepubertal human testes behave similarly in monolayer culture to adult rat testicular interstitial cells, viz. initial testosterone secretion being replaced by progesterone secretion. However, cultures prepared from human foetal and neonatal testes continue to produce testosterone in vitro (O'Hare et al., 1976a).

Unlike the adrenocortical cells in monolayer culture, addition of trophic hormones (HCG or cyclic AMP) to the cultures of testicular interstitial cells does not produce morphological changes in the cells. This is at variance with the results of Davis (1978) who reported that testicular cells in monolayer culture spontaneously produce tubules and spheres in the tissue culture flask. In our laboratory, culture conditions are arranged to suppress cell proliferation, whereas this is not so for the studies of Davis (1978). This probably explains the discrepancy.

Testes removed from some patients with testicular feminisation were available for study. Monolayer cultures prepared from these behaved like foetal testicular cells with prolonged testosterone production *in vitro*.

My interest in the function of the foetal rat testis at the time of sexual differentiation led me to postulate that progesterone may be an important testicular secretory product involved with Mullerian duct inhibition (*vide supra*). My organ culture studies gave circumstantial evidence that progesterone had a deleterious effect on the Mullerian duct. During the course of these experiments I had gained experience at dissecting the small rat foetal testes at the time of early gonadal differentiation. It was therefore logical that I should next prepare monolayer cultures of foetal rat testes and examine the daily output of endogenously synthesised steroids. I prepared cultures from foetal rat testes ranging in gestational age from 14½ to 20½ days, and collected and replaced the culture medium daily. I then examined the daily steroid output by radioimmunoassay and high pressure liquid chromatography over a 20 day period in monolayer culture, during which time I stimulated the cells with HCG or cyclic AMP. I used the testosterone radioimmunoassay already developed in the laboratory, and I developed a similar assay for progesterone. The high pressure liquid chromatograph was a recent aquisition to our laboratory and many of the parameters required to give optimal steroid separation had to be established.

The results of these experiments are presented in Chapter 4.

SECTION I : EXPERIMENTAL STUDIES

CHAPTER 2

MATERIALS AND METHODS

CHAPTER 2

IN VITRO STUDIES: MATERIAL AND METHODS

A. ANIMALS

1. Strain of rats

Wistar rats with a gestational period of approximately 22 days, and an oestrous cycle of 5 days, were used in the present experiments.

2. Preparing foetal rats of known gestational age

An excess of mature virgin female rats were housed overnight with males and separated the following morning whether or not there was evidence of copulation. This was designated as day ½ . After 7-10 days, pregnancy could be detected by palpation of the mothers, and non-pregnant rats were used for further mating.

B. TISSUE CULTURE TECHNIQUES

Organ culture and monolayer techniques were developed for the experimental part of this thesis. Foetal rat genital tracts were explanted and maintained whole in organ culture. In other experiments, the cells of foetal rat testes were dissociated into single cell suspensions and subsequently maintained in monolayer cultures.

1. Organ culture of foetal rat genital tracts

Foetal rat genital tracts were cultured either on solid culture medium containing 0.8% agar, or on stainless steel grids overlying liquid culture medium. The constituents of the culture media used are shown in Table 2.I and the preparation of these media is presented in Appendix VII.

a) Addition of steroid or polypeptide hormones to culture medium

Solutions of testosterone or pregesterone in ethanol, and HCG (Human chorionic gonadotrophin: "Pregnyl", Organon) in sterile saline were prepared 100 times the final concentration required, and 200µl was added to 20ml of culture medium as required. Because addition of steroids resulted in 1% ethanolic solution in the culture medium, a similar concentration of ethanol was used in control cultures. However, a 1% concentration of ethanol was found to be toxic to some cultured genital tracts, and subsequently more concentrated stock steroid

Table 2.I. Concentrations of constituents of tissue culture media used for organ culture of foetal rat genital tracts on solid media.

Constituent	Medium B	Medium C
Medium 199 (with Hank's salts)	X 1	X 1
HEPES buffer	20 mM	20 mM
Agar	0	0.8 %
Bicarbonate	0.56 g/l	0.56 g/l
Foetal calf serum	20 %	20 %
Penicillin / streptomycin	25 units/ml	25 units/ml
Neomycin	50 μg/ml	50 μg/ml
Amphotericin	1.25 μg/ml	1.25 μg/ml

steroid solutions in ethanol were prepared so that the desired concentration of steroid in the medium could be obtained without producing a concentration of ethanol greater than 0.2% in the culture medium.

The following final concentrations of hormone were used in the experiments:-

Progesterone	10^{-5}M; 5X 10^{-5}M; 10^{-4}M
Testosterone	10^{-4}M
HCG	10 iu/ml

b) Dissection of foetal rat genital tracts

Pregnant Wistar rats at 14½ to 17½ days gestation were killed by cervical dislocation and the uteri, containing 5 to 15 foetuses, were transferred to a large sterile Petri dish. Each embryo, with placenta and foetal membranes intact, was removed in turn from the uterus and transferred to a sterile Petri dish. Under a Wild M5 stereoscopic dissecting microscope, the genital tracts were exposed, carefully dissected free, and placed on a square of Millipore filter approximately 0.5cm in length. In some instances, the gonads were removed from the genital tracts before the genital tracts were dissected free. By 14½ days gestation, the foetal testis is larger and more vascular than the ovary, and the two are readily differentiated under the dissecting microscope. All dissecting instruments were sterilised before use, and aseptic techniques were used throughout.

c) Maintaining rat foetal genital tracts in organ culture

When using solid tissue culture medium, the Millipore filter with genital tract was laid on top of the solid ridge of medium C, and approximately 2ml of medium B was placed in the Petri dish around the gel to keep it moist. Any substances added to medium C (e.g. steroids or HCG) were also added to medium B. The Petri dishes with genital tracts were loosely covered and incubated at 37° C for 3 days in a covered sandwich box containing a little distilled water to reduce evaporation. The HEPES buffer maintains a physiological pH in an atmosphere of air.

In the liquid culture medium system, approximately 2ml of medium D was placed in the well of a Falcon tissue culture dish and a sterile steel wire grid was placed over the liquid so that the top of the fluid touched the bottom of the grid. The Millipore filter with genital tract was placed on the grid exposing the tissue to culture medium. The porous paper in the outer well of the tissue culture dish was moistened to reduce evaporation and the culture dishes were incubated at 37°C for 3 days in a covered sandwich box containing a little distilled water as before.

d) Assessing genital duct development or regression in organ culture

It was assumed that all the foetuses in one litter had reached to same stage of genital duct development at the time of the start of the tissue culture. One male and one female genital tract were dissected free, placed on Millipore

filter and immediately fixed in Bouin's fixative to give an indication of the stage of development at the time of explanation. After 3 days in organ culture, the remaining genital tracts on Millipore filter were fixed in Bouin's fixative. Because the genital tracts are so small, complete fixation occured in 2 hours after which time the tissue was transferred to 70% ethanol to prevent over-fixation which made the delicate tissue brittle and difficult to section. The genital tracts and Millipore filter were embedded together in paraffin wax with the Millipore perpendicular to the face of the block. The block was then sectioned serially at 10 microns through the whole length of the genital tracts, each 10th section being mounted, stained with haematoxylin and eosin, and examined histologically. There were approximately 300 transverse sections of genital tract in each block, therefore the tissue was examined at 30 different levels. Each histological section contains 2 genital tracts (left and right) and each tract contains 2 genital ducts (Mullerian and Wolffian). The condition of each duct, and gonad if still attached, was assessed histologically at every level so that the extent of *in vitro* development or regression could be determined.

Using this system, the effects of castration (removal of gonads before explanting the genital tracts), or the effect of added hormones, can be studied *in vitro*.

2. Monolayer culture of foetal testes

The preparation and constituents of the culture media used in these experiments are shown in Appendix VII.

Preparation of monolayer cultures

Foetal rat testes contain histologically recognizable interstitial (Leydig) cells from 14½ days gestation onwards (Fig. 2.1). Older foetal testes have relatively more seminiferous tubular tissue than interstitial tissue, but there is no reduction in absolute number of Leydig cells. It is assumed that the Leydig cells are mainly responsible for the steroidogenic activity of the foetal testes. No attempt was made to separate the interstitial and tubular tissue. Because foetal rat testes are so small, up to 40 testes were used to prepare a single monolayer culture. Therefore, foetal rats from up to 4 litters were required for each culture. Cultures were prepared from foetal rat testes ranging from 14½ to 20½ days gestation.

Two to 4 pregnant Wistar rats of identical gestational age were killed one at a time by cervical dislocation, and the uteri containing the foetuses removed aseptically and placed in sterile Petri dish. The genital tracts of each foetus were exposed aseptically under a dissecting microscope and the sex of the gonad determined. Female gonads were discarded. The larger more vascular testes were identified, removed, and stored in HEPES buffered "dissecting medium" at 4°C for up to 3 hours till all the testes were collected.

Fig. 2.1. Photomicrographs of foetal testes.

Fig. 2.1a. Human foetal testis (22 weeks gestation) showing solid primary sex cords consisting of primitive germ cells and Sertoli cells. There are many plump interstitial cells of Leydig between the sex cords. (H & E X 475).

Fig. 2.1b. Rat foetal testis (18½ days gestation). The features are similar to those of the human foetal testis with solid primary sex cords and many Leydig cells in the interstitium. (H & E X 475).

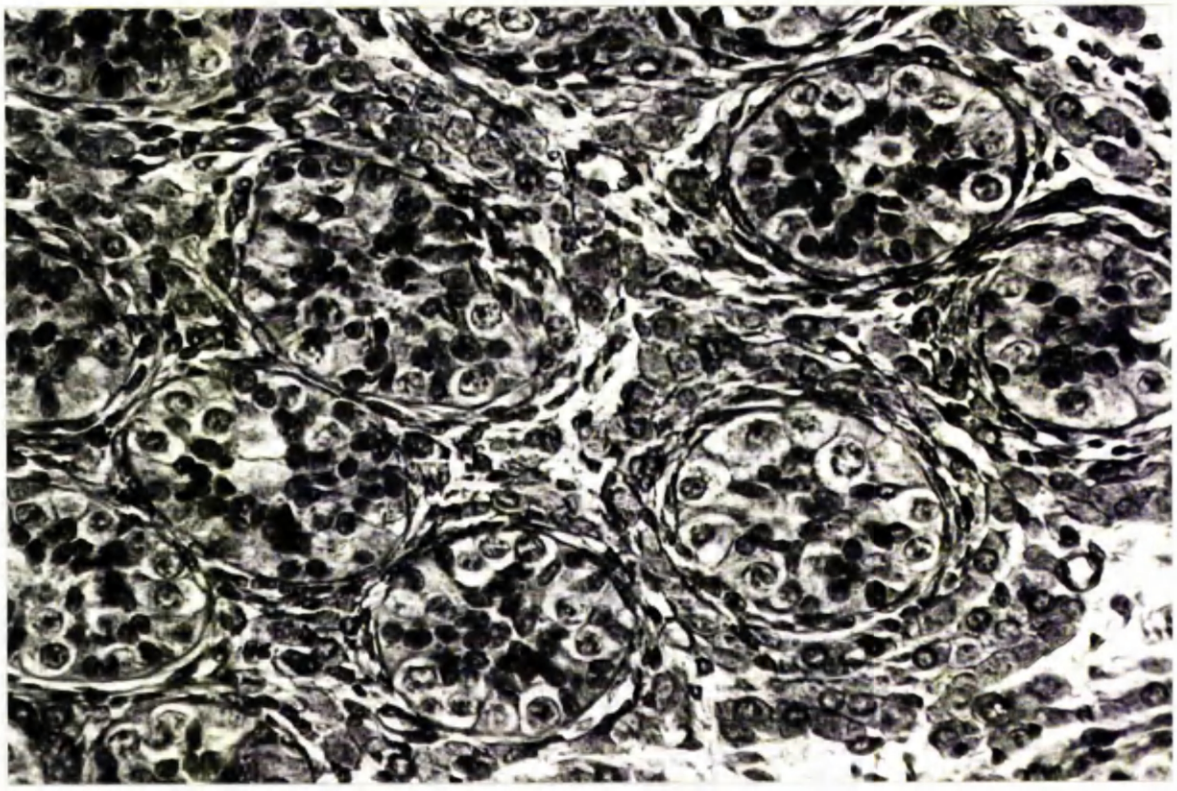


Fig. 2.1a.

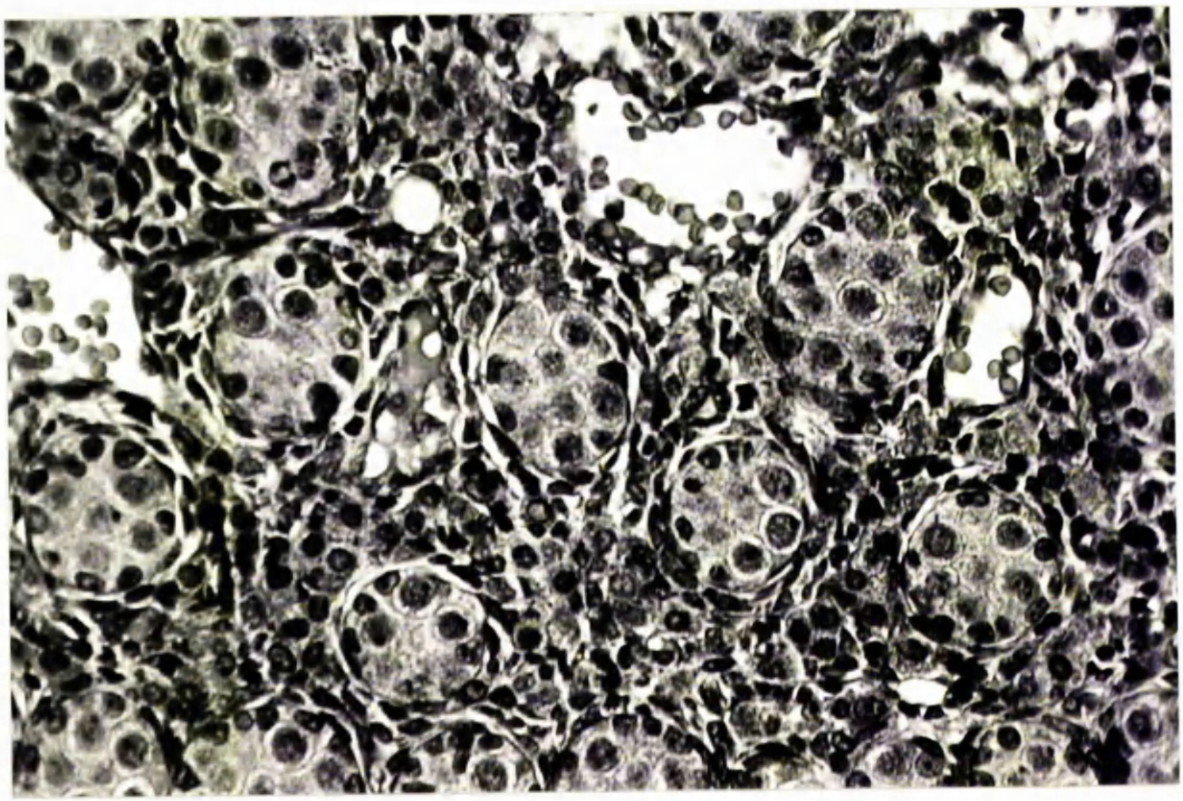


Fig. 2.1b.

Fig. 2.1. Photomicrographs of foetal testes.

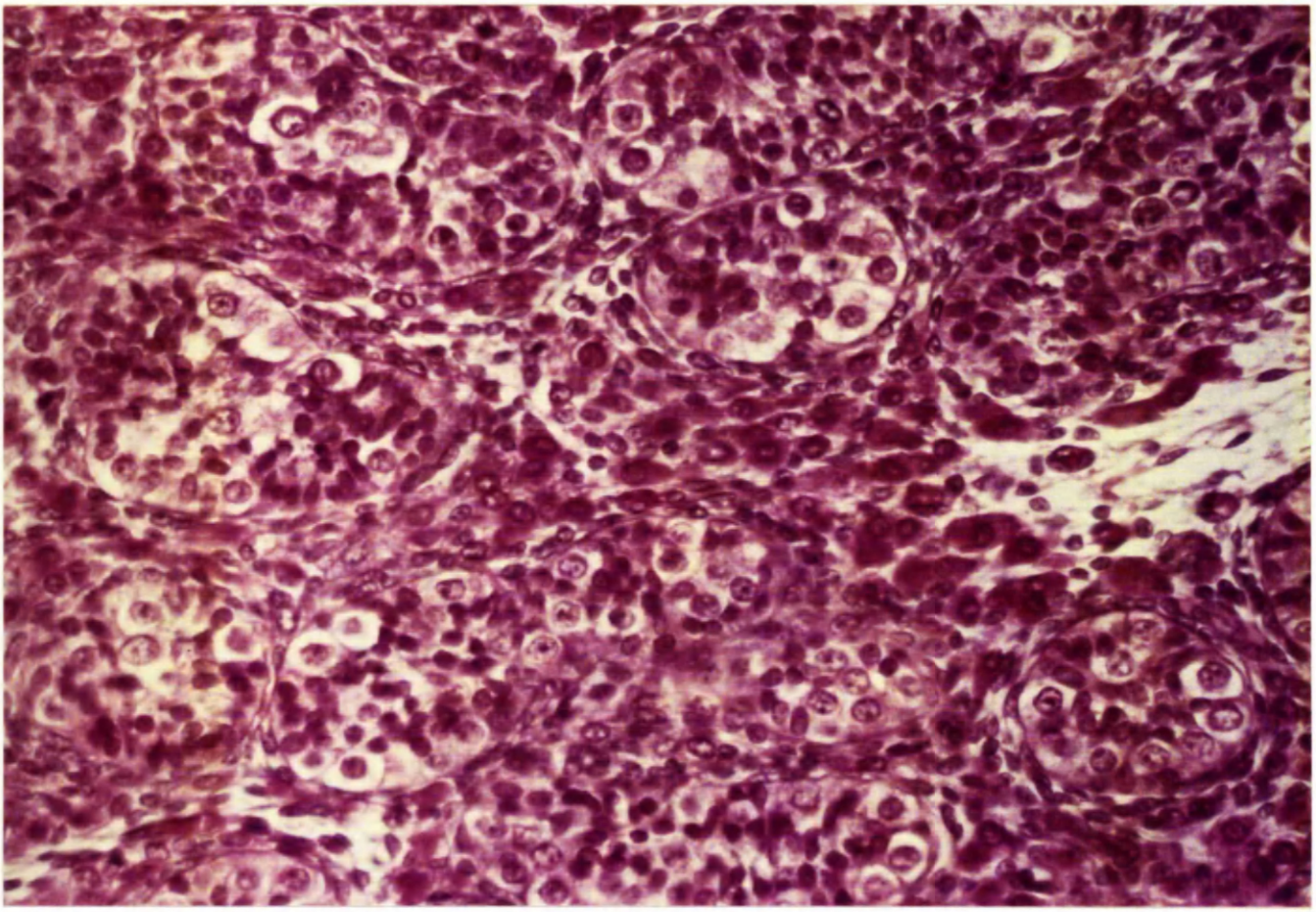


Fig. 2.1c. Colour photomicrograph of human foetal testis (22 weeks gestation; same as Fig. 2.1a). (H & E X 450).

When all the testes had been removed, each was cut into 4-5 pieces under the dissecting microscope in "dissecting medium", and transferred to a sterile universal container where they were allowed to settle to the conical apex. The supernatant "dissecting medium" was sucked off with a Pasteur pipette and replaced by 5ml of enzyme solution" (hyaluronidase and collagenase in "dissecting medium") at 37°C. After 2 hours incubation at 37°C the "enzyme solution was sucked off and discarded, and replaced by 5ml of "dissecting medium". The testes and medium were vigorously agitated approximately 10 times with a Pasteur pipette, and allowed to settle for 2 minutes during which time undissociated clumps of testicular tissue and capsule sedimented to the bottom,while dissociated cell remained in suspension. The supernatant medium containing dissociated cells was transferred to a clean sterile universal container and the remaining clumps of tissue were again agitated 10 times in 5ml of "dissecting medium" using a Pasteur pipette. After 2 minutes settling time, the supernatant cell suspension was removed and pooled with the cells from the first dissociation. The clumps were agitated a third time in "dissecting medium", and the cells suspension (after settling for 2 minutes) again pooled with the previous dissociations. It was assumed that the 15ml pool of dissociated cells contained most of the interstitial and tubular cells, whereas solid clumps of capsule and undissociated testicular tissue were discarded in the sediment.

The 15ml cell suspension was gently centrifuged for 5 minutes in a MSE bench centrifuge at a setting of 2½. The supernatant was decanted and discarded, and 5ml of bicarbonate buffered "cultured medium" added to the pellet at the bottom of the universal container.

The cells in the pellet were resuspended by gentle agitation with a Pasteur pipette for approximately 1 minute, transferred to a Falcon tissue culture flask, gassed for 30 seconds with 10% CO_2 in air (BOC), tightly stoppered, and incubated at 37°C.

After 24 hours incubation, many cells had adhered to the foot of the flask, and others remained in suspension. It is thought that viable Leydig and Sertoli cells adhere to the bottom of the flask while non-viable cells and the less dense germ cells remain in suspension. The supernatant fluid was decanted into a stoppered 5ml plastic Bijoux bottle (Falcon) and stored at -20°C. Five ml of fresh "culture medium" was pipetted into the tissue culture flask, then gassed with 10% CO_2 in air, stoppered and replaced in the 37°C incubator. The culture medium was changed and collected daily. After 10 days in a non-hormonal environment, the monolayer culture was maintained for a further 5 days in the presence of culture medium containing 50 iu HCG per 5ml (changed daily), then a further 5 days in culture medium containing 210µg monobutyryl cyclic adenosine monophosphate (cAMP: Sigma) per ml (0.5mM).

Each culture was examined daily, with a Wild M40 phase-contrast inverted microscope to check viability of culture cells, plating density, and to ensure that there was no bacterial overgrowth. The appearances of a typical culture is shown in Fig. 2.2.

One culture was prepared from twenty 18½ days rat foetal testes, the total weight of the testes being approximately 8mg. No other attempt was made at quantitation for several reasons:-

a) It was difficult to remove excess fluid and weigh the foetal testes without jeopardising cell viability by dehydration.

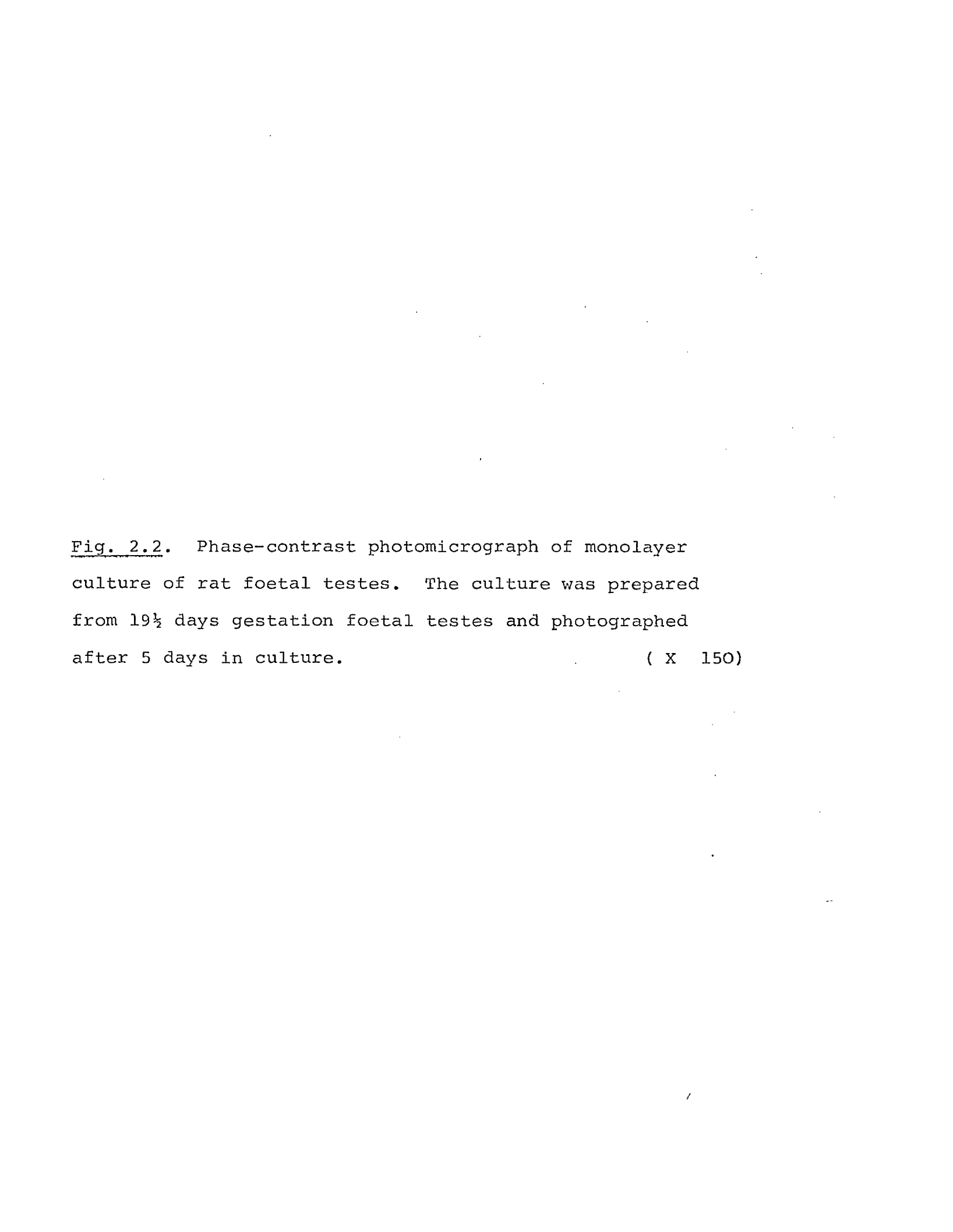


Fig. 2.2. Phase-contrast photomicrograph of monolayer culture of rat foetal testes. The culture was prepared from 19½ days gestation foetal testes and photographed after 5 days in culture. (X 150)

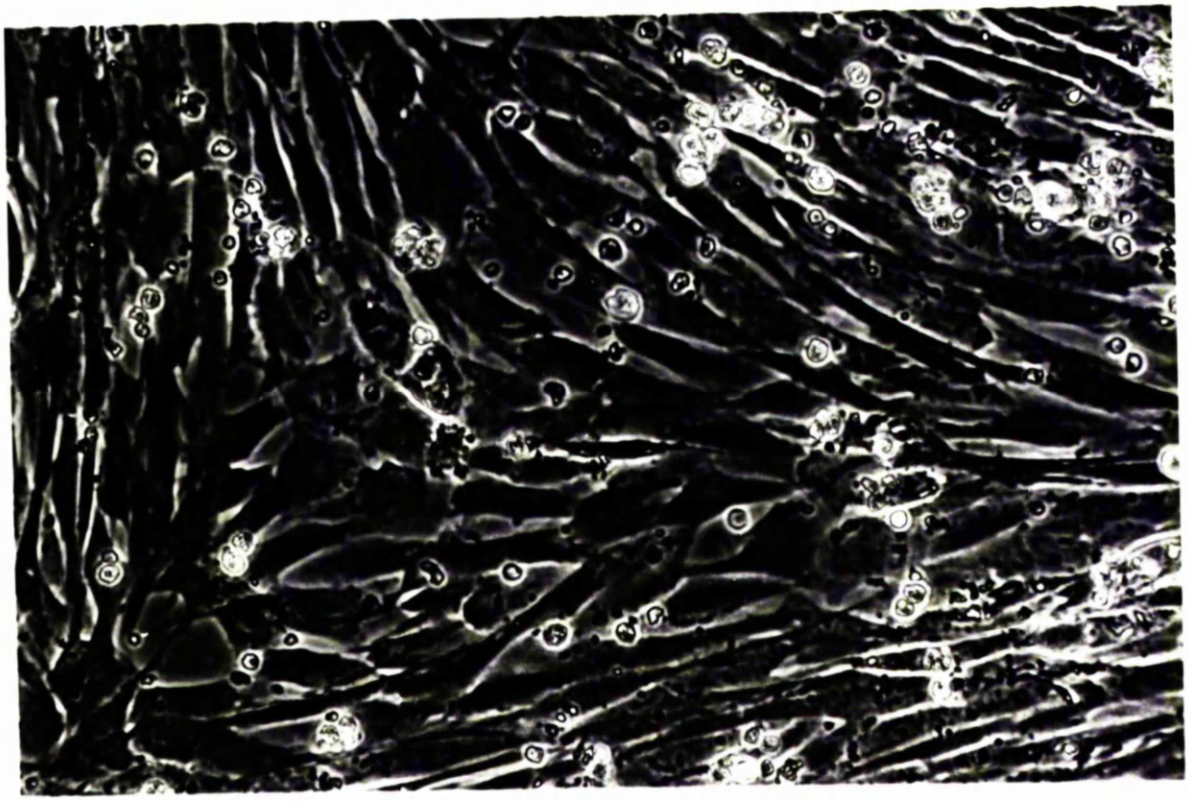


Fig. 2.2

b) Not all the testicular tissue was used for cell culture. The capsule, undissociated clumps of testes, damaged cells and probably germ cells were discarded.

c) The proportion of Leydig cells in the testis varied greatly with gestational age, therefore the proportion of steroidogenically active cells is unknown.

d) A cell count was not feasible because of marked variability of plating density within single cultures.

e) The number of cells in each culture was not static. In some cases there was gradual cell loss but in some others definite proliferative activity occurred.

C. ASSAY OF CULTURE MEDIA

1. High pressure liquid chromatography (HPLC):

Description and separating capacity

A Du Pont model 830 high pressure liquid chromatograph (Du Pont, Wilmington, Dela., USA), equipped with a model 837 variable wavelength spectrophotometer, and a model 838 programmable gradient elution module, was used to detect endogenous steroid production from the rat testes cells in monolayer culture. Extracted samples were injected via a Rheodyne septumless valve. The spectrophotometer was operated in the double beam mode with an air reference cell, and chromatographs were recorded in a Phillips PM 8000 flat-bed recorder. The apparatus is shown in Fig. 2.3.

Steroids are relatively non-polar compounds and are best separated by liquid-liquid partition column chromatography. The stationary liquid phase is a non-polar liquid which absorbs steroid molecules. This liquid is chemically bonded to inert silaceous particles which are packed into a column. The silaceous particles are small in order to increase the surface area of a small volume of stationary phase, and therefore to increase the efficiency of separation. The absorbed steroids are eluted from the column by gradient elution using a polar mobile phase liquid. The flow rate of the mobile phase is increased by applying it to the column under very high pressure (up to 4,500 pounds per square inch), and by heating the column to 40-45^{o}C in order to reduce viscosity. Further increase in temperature causes increased activity of the porous sites and increased polarity of the stationary phase thus increasing retention volume.

Fig. 2.3. Photograph of Du Pont Model 830 high pressure liquid chromatograph.

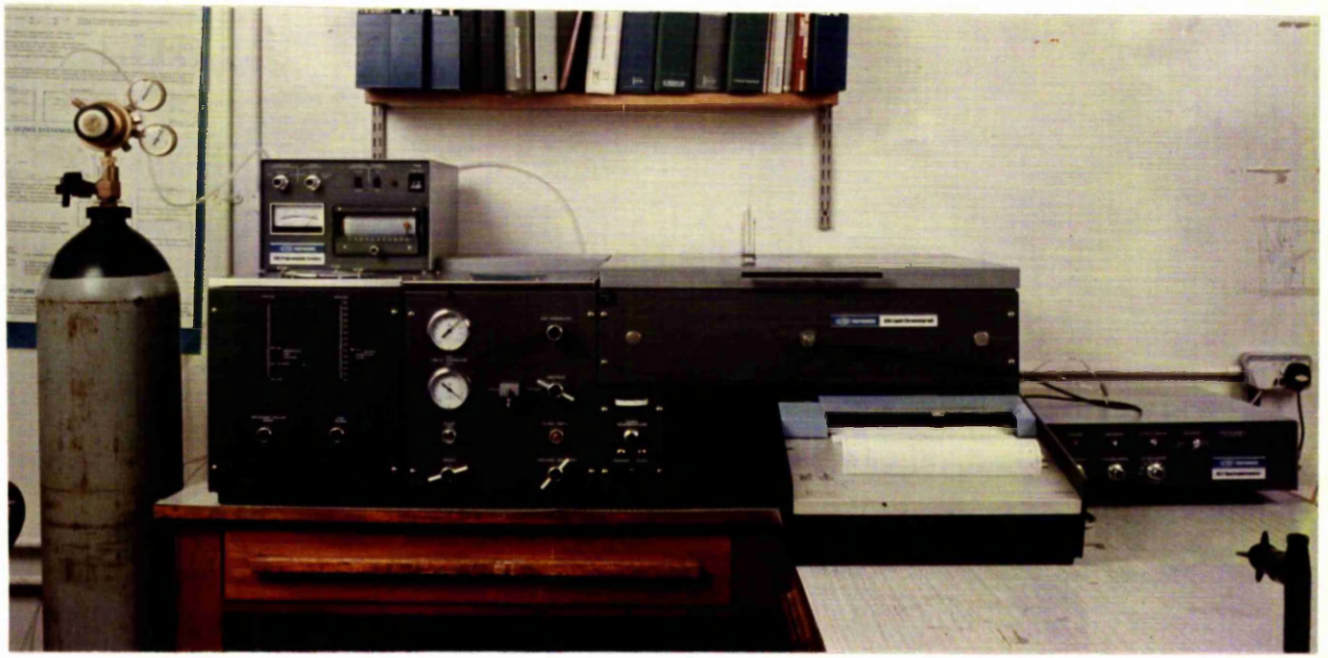


Fig. 2.3.

An optimum balance between flow rate and retention volume is determined experimentally in order to achieve maximum resolution without thermal instability.

HPLC has the advantages of speed, versatility, high sensitivity, good resolution, and both quantitative and qualitative analysis of steroids without their destruction. The steroids can therefore be recovered at the end of the separation. Small bore columns packed with small sized particles give best results in high speed chromatographic separations (Brown, 1973). HPLC is better than thin layer chromatography (TLC) which is time-consuming, lacks sensitivity, and is difficult to quantitate. HPLC is also superior to gas liquid chromatography (GLC) in which the steroids often required to be derivativised prior to application to the column so that they can be applied in a gaseous phase.

In the present experiments, the steroids produced by the foetal rat testes in monolayer culture and released into the tissue culture media were extracted and analysed by HPLC. Gradient elution with 2 different solvent systems (acetonitrile/water and dioxane/water) were used. The method of extracting steroids from the tissue culture media for HPLC, and the full HPLC procedure, are given in Appendix IX. The capacity to separate different standard steroids is shown in Figs. 2.4,&2.5 and the retention times of 47 different steroids are given in Table 2.II. The resolving power of HPLC to separate the steroids produced by the foetal testes in monolayer culture is shown in Table 2.III. It was found that in the HPLC systems used in our laboratory, the peak height in the chromatogram is linearly proportional to the mass of steroid applied to the

Fig. 2.4. Separation of steroid standards (330-750 ng) by high pressure liquid chromatography. The standard steroids used are those expected to be produced by the testis in monolayer culture. (From O'Hare et al., 1976b).

------ solvent gradient ——— optical density at 240nm

Chromatographic conditions:

Column:	Zorbax-ODS
Solvent (start):	32% (v/v) acetonitrile:water
Solvent (finish):	100% acetonitrile
Gradient:	$y = x^3$
Time:	50 minutes
Temperature:	$45^{o}C$
Pressure:	2,000 lbs/square inch
Flow rate (start):	0.38 ml/min
Flow rate (finish):	0.80 ml/min
Attenuation:	0.32 absorption units full scale

Abbreviations (full systematic nomenclature - Appendix I):

AD:	Androstenedione
DHA:	Dehydroepiandrosterone
DHP:	20α-Dihydroprogesterone
DHT:	5α-Dihydrotestosterone
17α-OHP:	17α-Hydroxyprogesterone
17α-OH DHP:	17α-Hydroxy-20α-dihydroprogesterone
16α-OHP:	16α-Hydroxyprogesterone
P:	Progesterone
7α-OHT:	7α-Hydroxytestosterone
T:	Testosterone

Chromatogram of testis steroid standards

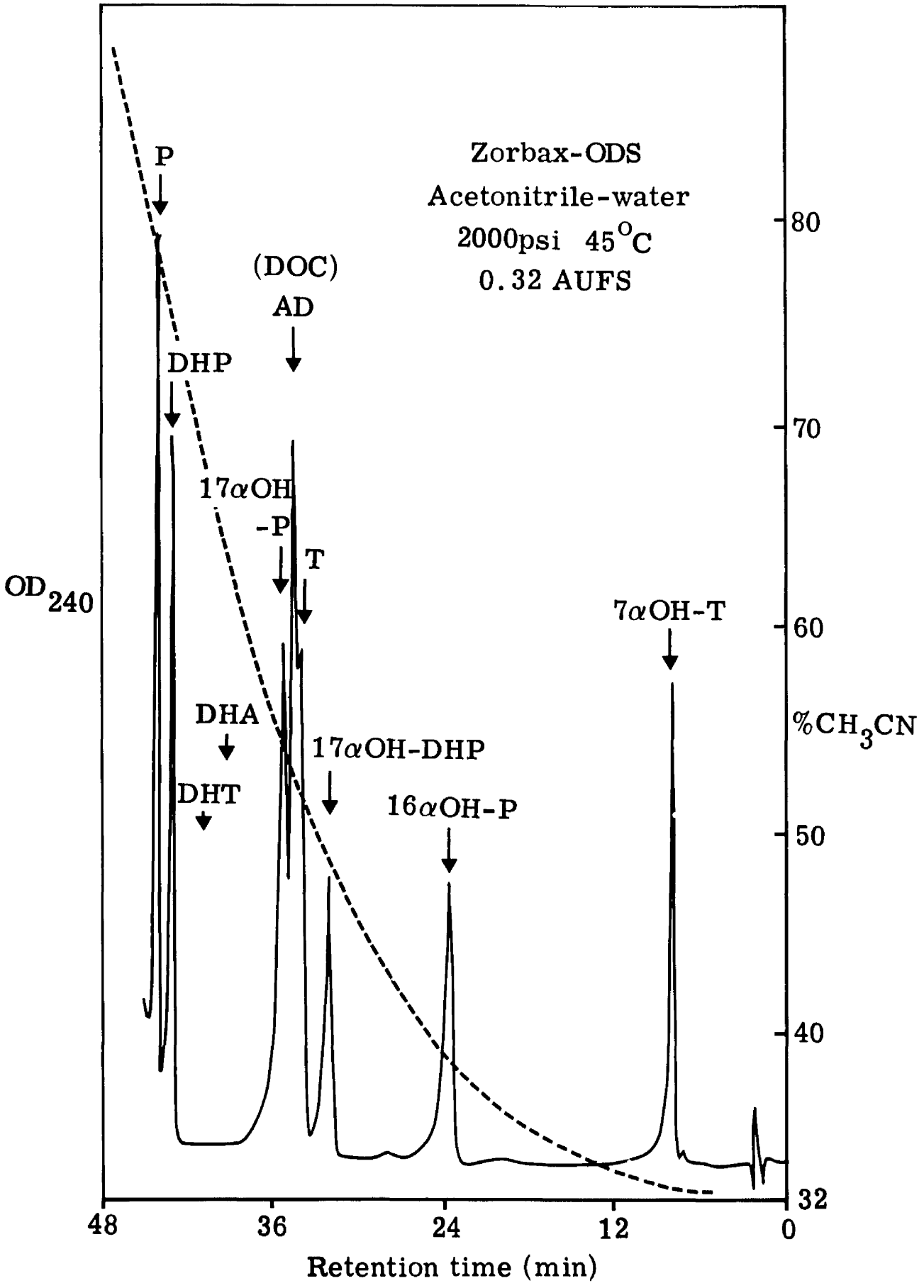


Fig. 2.4.

Fig. 2.5. Separation of polar steroid standards (250-750ng) by high pressure liquid chromatography. The standard steroids used are those expected to be produced by the adrenal in monolayer culture. (From O'Hare et al., 1976b).

------ solvent gradient ——— optical density at 240nm

Chromatographic conditions:

Column:	Zorbax-ODS
Solvent (start):	20% (v/v) dioxane:water
Solvent (finish):	100% dioxane
Gradient:	$y = x^3$
Time:	50 minutes
Temperature:	$45^{o}C$
Pressure:	2,500 lbs/square inch
Flow rate (start):	0.38 ml/min
Flow rate (finish):	0.34 ml/min
Attenuation:	0.16 absorption units full scale

Abbreviations (full systematic nomenclature - Appendix I):

ALDO:	Aldosterone
B:	Corticosterone
F:	Cortisol
E:	Cortisone
DHALDO:	11-Dehydroaldosterone
11βOH-AD:	11β-Hydroxyandrostenedione
18OH-B:	18-Hydroxycorticosterone
6βOH-F:	6β-Hydroxycortisol
18OH-A:	18-Hydroxy-11-dehydrocorticosterone
18OH-DOC:	18-Hydroxy-11-deoxycorticosterone

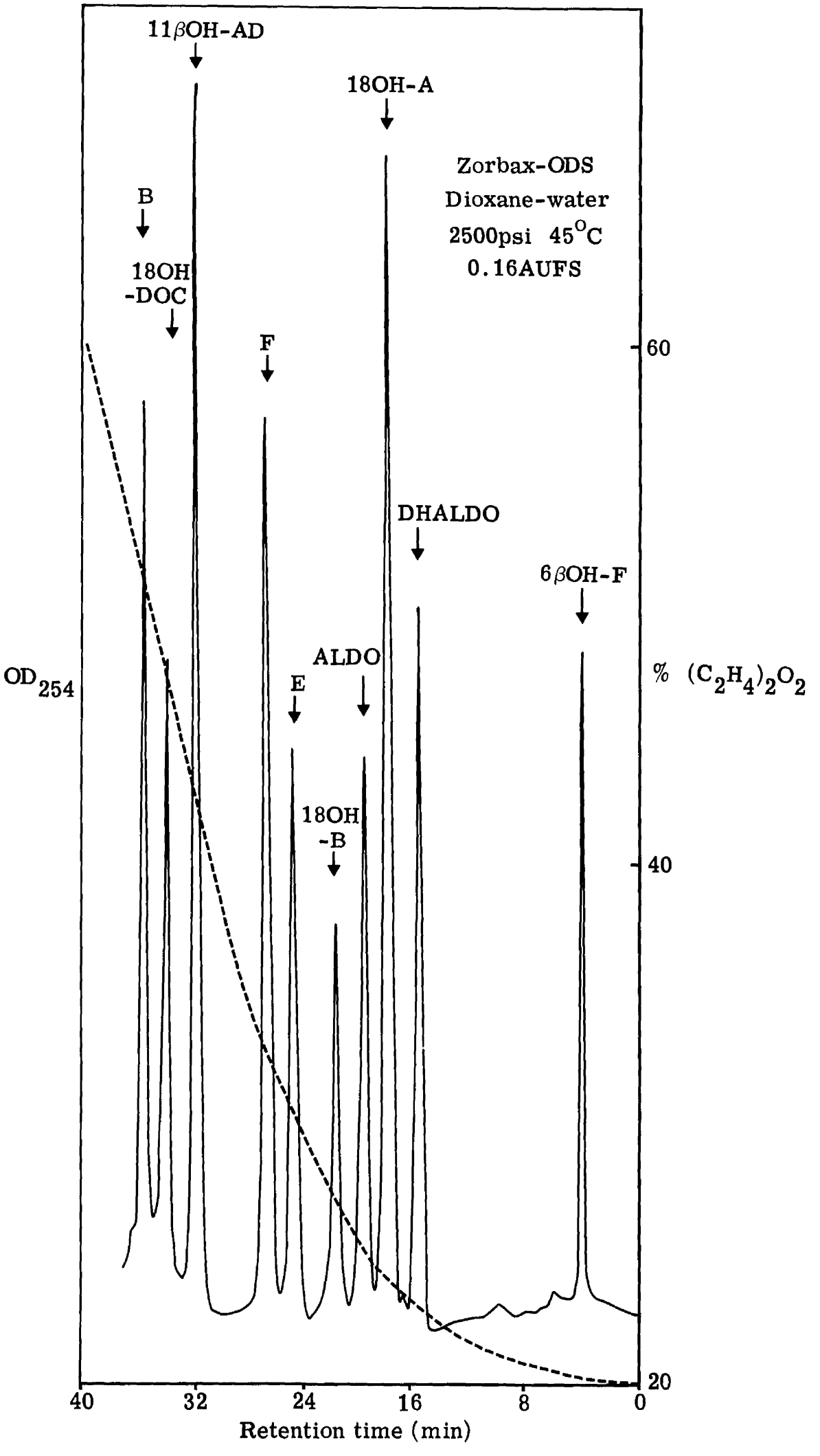

11βOH-AD
18OH-A
Zorbax-ODS
Dioxane-water
2500psi 45°C
0.16AUFS
B
18OH
-DOC
F
DHALDO
6βOH-F
ALDO
E
18OH
-B
OD254
% (C2H4)2O2
60
40
20
40
32
24
16
8
0
Retention time (min)

Table 2. II. Retention times in minutesfor 47 standard steroids on Zorbax - ODS using acetonitrile or dioxane as mobile phase.

Steroid *	Retention time (min)	
	Acetonitrile (32 - 100 %)	Dioxane (20 - 100 %)
6β-hydroxycortisol	3.5	4
11-dehydroaldosterone	5	15
17-isoaldosterone	6	15
18-hydroxy-11-dehydrocorticosterone	6.5	17.5
prednisone	9	23
aldosterone	8	19
oestriol	8	25
cortisone	9	25.5
19-hydroxyandrostenedione	11.5	20
17α-hydroxytestosterone	9	19.5
18-hydroxycorticosterone	7	21
prednisolone	9	26.5
cortisol	9	27
adrenosterone	17	28
19-hydroxytestosterone	10	24
16α-hydroxytestosterone	11	27
11-dehydrocorticosterone	15	29.5
dexamethasone	13	35
11β-hydroxyandrostenedione	17	32
21-deoxycortisol	16	34.5
corticosterone	18	36
11β-hydroxytestosterone	15	33.5
18-hydroxydeoxycorticosterone	16	34.5
11-deoxycortisol	20	37.5
6α-hydroxyprogesterone	24.5	38.5
16α-hydroxyprogesterone **	24	38
11-ketoprogesterone	30	40

Steroid *	Retention time (min)	
	Acetonitrile (32 - 100 %)	Dioxane (20 - 100 %)
oestrone	30. 5	42. 5
11β-hydroxy-20α-dihydro-progesterone	26	39
androstenedione **	36	42
6β-hydroxyprogesterone	30	41
oestradiol	29	43
11β-hydroxyprogesterone	33	43
11-deoxycorticosterone	36	43. 5
17α-hydroxyprogesterone **	38	45
testosterone **	35	43. 5
17α-hydroxy-20α-dihydro-progesterone **	32	42
17α-hydroxy-20β-dihydro-progesterone	32. 5	43. 5
dehydroepiandrosterone	41	44
androstenediol	38	-
17α-hydroxypregneneolone	39	-
5α-dihydrotestosterone	42	-
progesterone **	45	49
20α-dihydroprogesterone **	43. 5	47. 5
20β-dihydroprogesterone	46	49. 5
pregnenolone	47	50
cholesterol	>60	-

* Systematic names of steroids are given in Appendix I

** These steroids were detected in foetal testes monolayer culture media. A standard solution containing a mixture of these 7 steroids was applied to the HPLC daily to check retention times.

Table 2. III. Retention times in minutes of standard steroids corresponding to steroids produced by foetal rat testes in monolayer culture.

Steroid *	Retention time (min)	
	Acetonitrile (32 - 100 %)	Dioxane (20 - 100 %)
16α-hydroxyprogesterone	24	38
androstenedione	36	42
17α-hydroxyprogesterone	38	45
testosterone	35	43. 5
17α-hydroxy-20α-dihydro-progesterone	32	42
progesterone	45	49
20α-dihydroprogesterone	43. 5	47. 5

* Nomenclature of steroids is given in Appendix I.

column (Fig. 2.6) over the range 40-2000 ng (O'Hare et al., 1976).

HPLC is less sensitive than radioimmunoassay (RIA) for detecting steroids, and cannot cope with large numbers of samples. In the present studies, HPLC was used to verify the nature of the steroids detected by RIA, and helped in their quantitation. HPLC was also used to detect steroids not measured by RIA.

2. Radioimmunoassay (RIA) of steroids

Radioimmunoassay of steroids has been possible since antisera to seroids have been available. Antibodies can be raised to steroids which have been coupled to antigenic proteins, and these form the basis of highly sensitive and specific radioimmunoassays (Cameron & Scarisbrick, 1973). All culture media collected from foetal rat testicular monolayer cultures were assayed by RIA for progesterone and testosterone.

Antiserum to progesterone-11α-hemisuccinyl-bovine serum albumin was raised in rabbits and kindly donated by Dr. K. McNatty; and antiserum to testosterone-3-oxime-bovine serum albumin was raised in rabbits and kindly donated by Mr. M. Mansfield. Anti-progesterone serum was diluted 1 in 5 in PBS "assay buffer" (Appendix IV) and stored at -20°C in 0.5ml aliquots. Anti-testosterone serum was stored undiluted at -20°C in 0.5ml aliquots.

Fig. 2.6. Graph to show the relationship between mass of steroid applied to the column of the high pressure liquid chromatograph, and the peak height of the chromatogram. The peak height is linearly related to the mass injected over the range 40-2000ng for progesterone and testosterone.

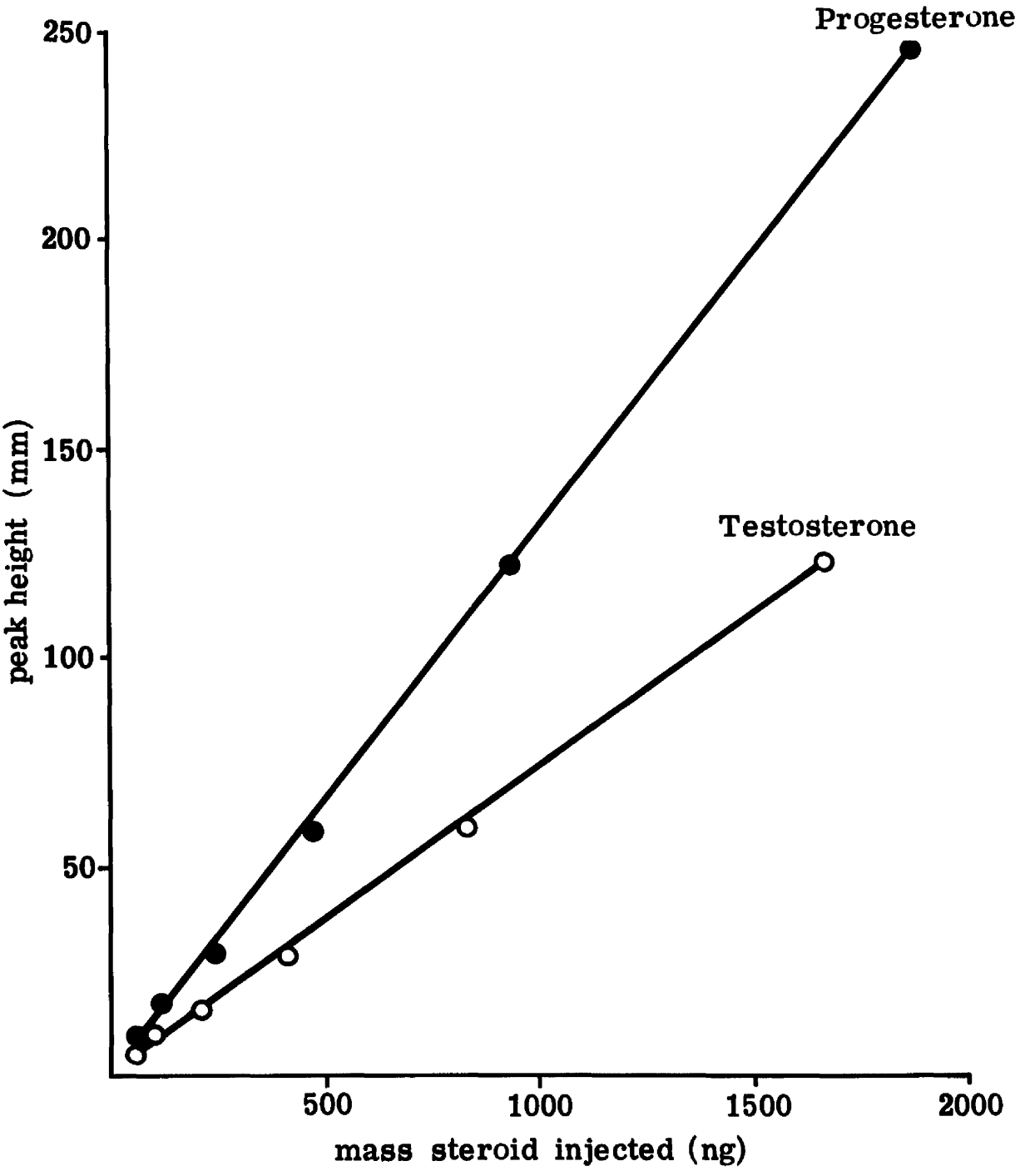

Progesterone
Testosterone
250
200
150
100
50
peak height (mm)
500
1000
1500
2000
mass steroid injected (ng)

Fig. 2.6.

Progesterone RIA

The progesterone RIA method used in the present series is similar to that described by Cameron & Scarisbrick (1973). The reagents, solutions and final procedure developed for the assay of progesterone in the tissue culture media are given in Appendix IV.

In the final stage of the assay, separation of free and bound progesterone was achieved using either dextran coated charcoal, which adsorbs free progesterone, or a solution of polyethylene glycol (PEG) which precipitates the progesterone bound to antibody. Figs. 2.7 (a&b) show standard curves for progesterone RIA using dextran coated charcoal and PEG. Fig 2.8 compares the use of dextran coated charcoal with PEG in the antibody dilution curves. These figures demonstrate that both dextran coated charcoal and PEG can be used effectively in the RIA of progesterone.

Dextran coated charcoal suspension is easier to pipette, and after centrifugation the supernatant is easier to decant than the more viscous PEG solution. Also PEG supernatant mixes less readily with Instagel. On the other hand the PEG solution does not require constant stirring before and during the pipetting stage(step vi), and is more stable to storage. Also, PEG is less liable to strip the labelled progesterone off the antibody than the dextran charcoal. If dextran charcoal is in contact with the antibody-bound progesterone for more than about 10 minutes, marked stripping of the label from the antibody occurs giving a falsely low reading for the percentage of label bound at the end of the incubation. For this reason, the total time taken to add the

Fig. 2.7. Standard curves for progesterone radioimmunoassay. The comparison of different agents used for separating free and bound progesterone is seen in Figs. 2.7a&b. Fig. 2.7c (over) shows a typical standard curve produced once optimal conditions had been established.

Fig. 2.7a. Dextran charcoal separation of free and bound progesterone after overnight incubation with antiserum. Each point is the mean of three readings, the individual values being represented by small dots.

Fig. 2.7b. Polyethylene glycol (PEG) separation of free and bound progesterone after overnight incubation with antiserum. Each point is the mean of three readings, the individual values being represented by small dots.

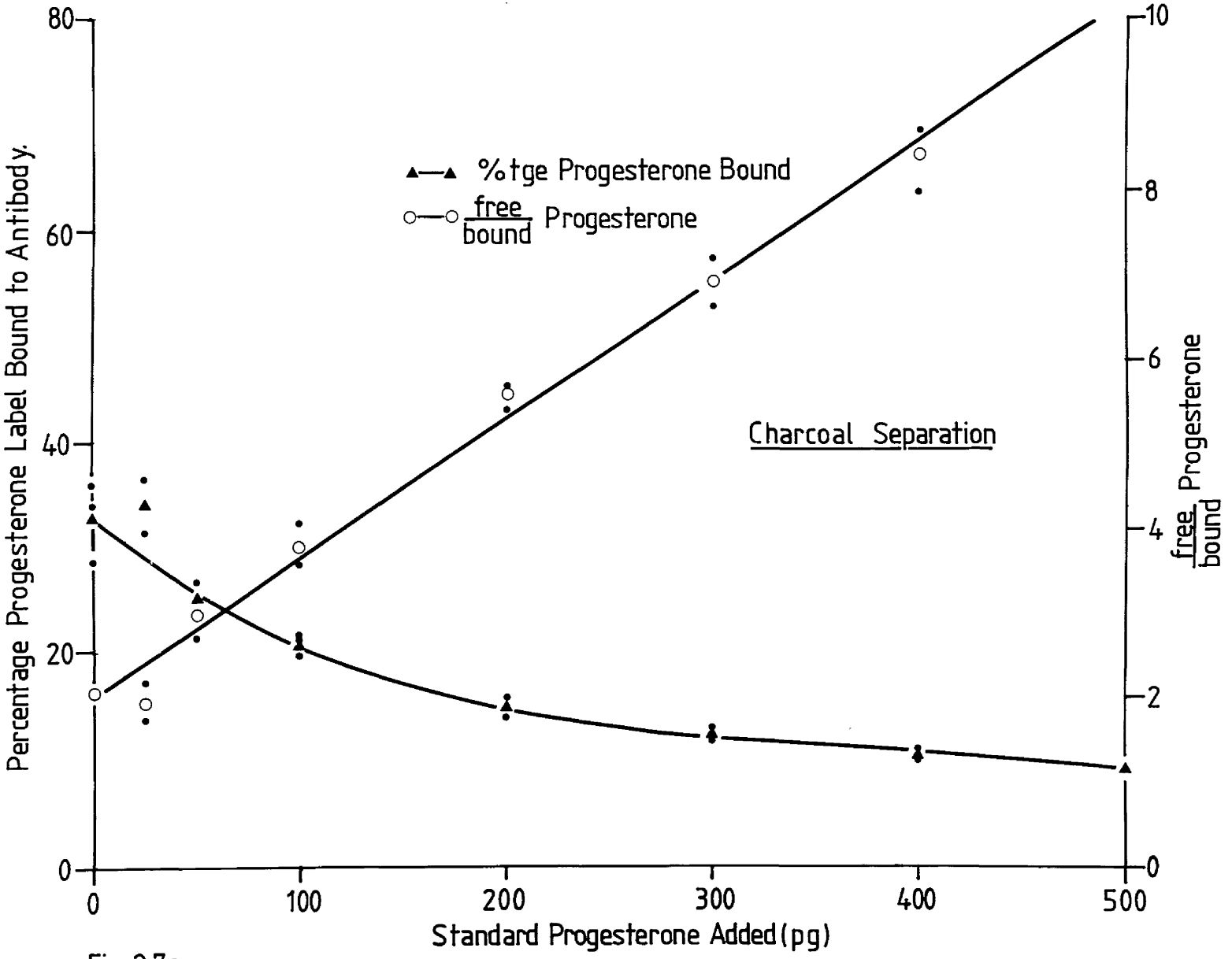

80
60
40
20
0
10
8
6
4
2
0
0
100
200
300
400
500
Percentage Progesterone Label Bound to Antibody.
free/bound Progesterone
Standard Progesterone Added (pg)
%tge Progesterone Bound
free/bound Progesterone
Charcoal Separation

Fig 2.7.a

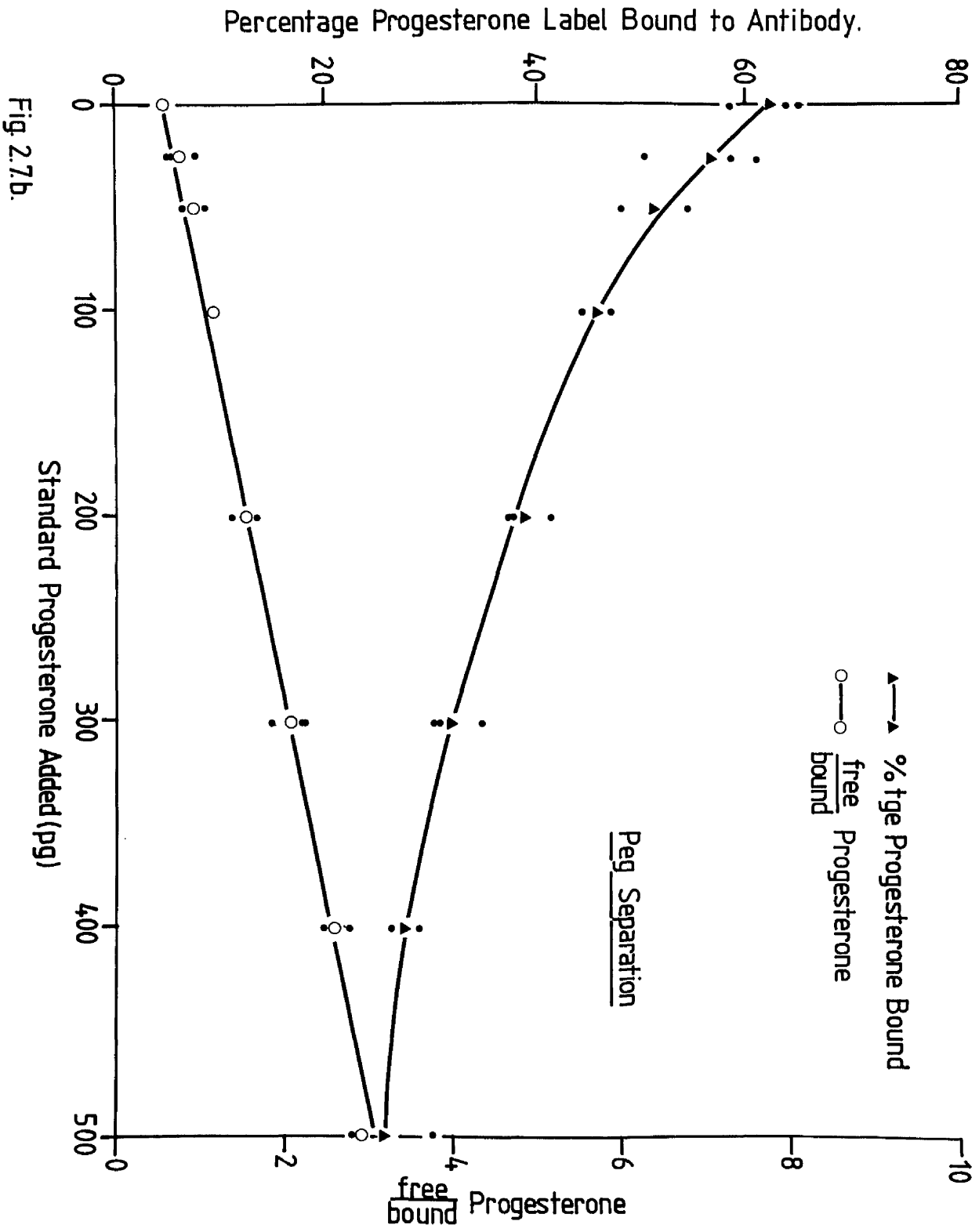

Percentage Progesterone Label Bound to Antibody.
0
20
40
60
80
0
100
200
300
400
500
Standard Progesterone Added (pg)
0
2
4
6
8
10
free/bound Progesterone
% tge Progesterone Bound
free/bound Progesterone
Peg Separation

Fig. 2.7b.

Fig. 2.7c. Standard curve for radioimmunoassay of progesterone using optimal parameters.

Standard steroid (in ethanol) dried under N_2 at 40°C

100µl of progesterone label (22,000 DPM) in assay buffer

100µl of 1/4000 antiserum in assay buffer

100µl of 0.5% gelatin in PBS

Incubated overnight at 4°C

1 ml of dextran coated charcoal suspension

Free and bound progesterone separated by centrifugation 5 minutes after dextran charcoal addition

Supernatant (with bound progesterone) added to 10ml of Instagel to detect radioactivity by scintillation counting of beta emissions.

Each point is the mean of four readings.

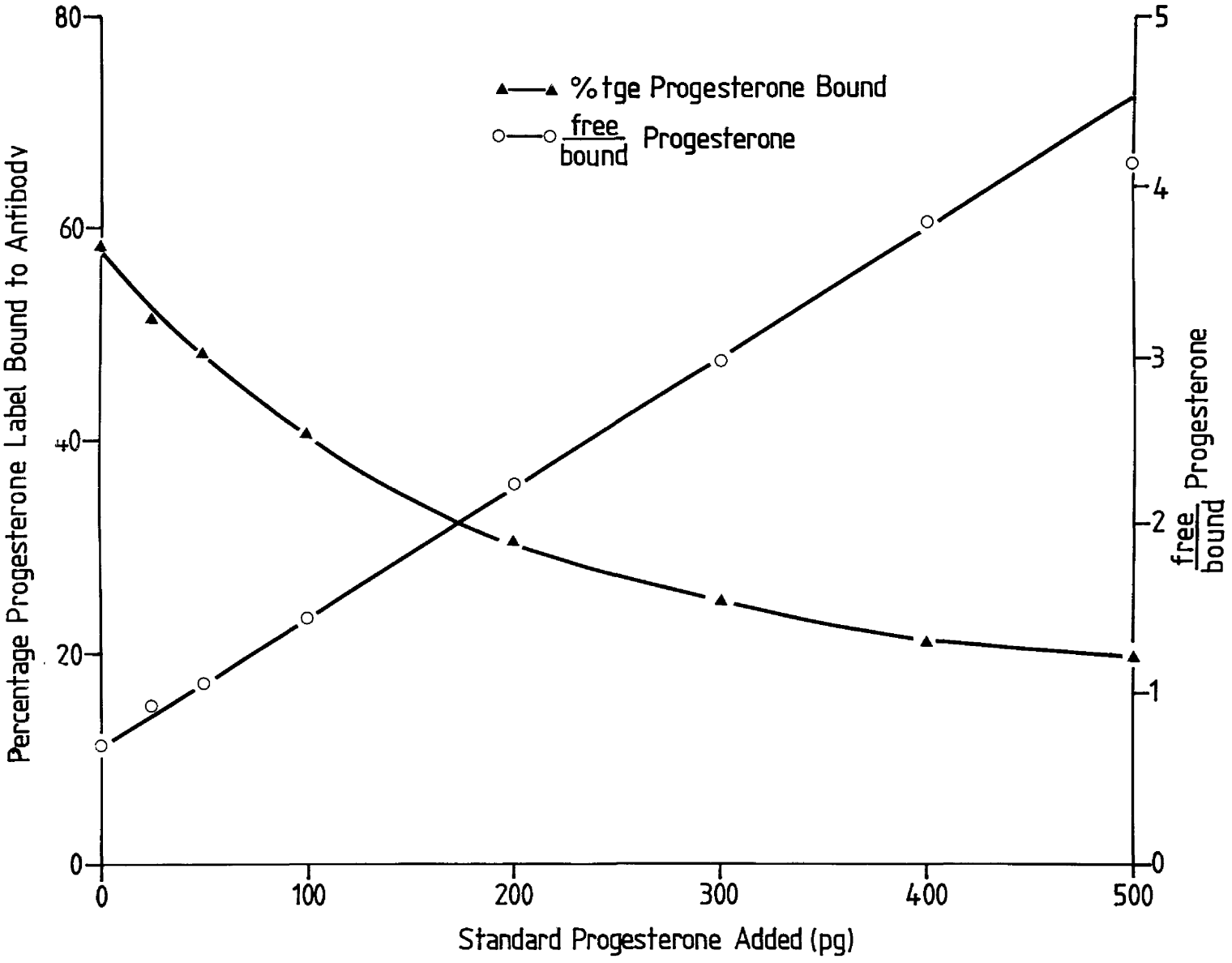
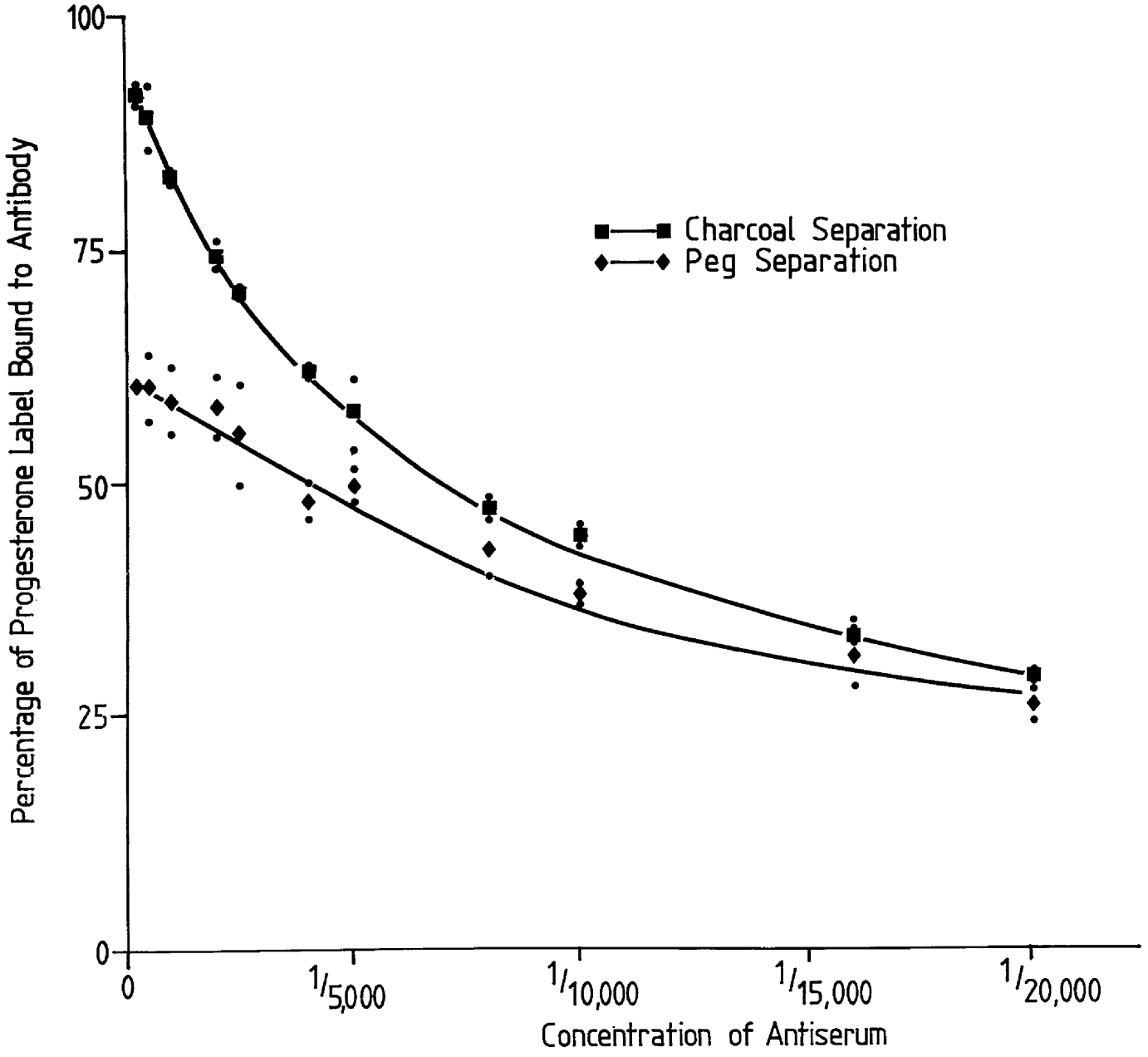

80
60
40
20
0
Percentage Progesterone Label Bound to Antibody
% tge Progesterone Bound
free/bound Progesterone
5
4
3
2
1
0
free/bound Progesterone
0
100
200
300
400
500
Standard Progesterone Added (pg)

Fig 2.7.c

Fig. 2.8. Progesterone RIA : antibody dilution curve. Comparison of dextran charcoal and PEG as agents for separating free and bound progesterone at the end of the incubation with antiserum.


100
75
50
25
0
Percentage of Progesterone Label Bound to Antibody
Charcoal Separation
Peg Separation
0
1/5,000
1/10,000
1/15,000
1/20,000
Concentration of Antiserum

dextran charcoal to all the tubes should not exceed 5 minutes (limiting the total number of assay tubes to about 80), and 5 minutes should elapse between addition of the dextran charcoal to the last tube and the start of the centrifugation. These times are less critical when using PEG because of the less severe stripping caused by PEG, therefore triplicate points show less scatter.

On balance, it was considered that dextran charcoal was more convenient than PEG. The precision of the Assay was therefore determined by the amount of stripping between the addition of the dextran charcoal and the separation of free and bound progesterone by centrifugation. Stripping was lessened by keeping the tubes at $4^{o}C$.

The dilution of antiserum used should give approximately 50% binding when no unlabelled progesterone is added, and less than 20% binding in the presence of 500pg progesterone. Fig. 2.8 shows that when using dextran charcoal a dilution of 1:6000 of anti-progesterone serum is suitable. The concentrated antiserum was stable when frozen, but the diluted (1:100) stock serum deteriorated slowly at $4^{o}C$ and less dilute antiserum was required in later experiments.

In the standard curves for progesterone RIA (Fig.2.7), plotting the percentage of labelled progesterone bound to antibody against the amount of unlabelled progesterone added gives aconcave curve on linear axes. The amount of progesterone in an unknown sample can then be determined by extrapolation. Alternatively, plotting the ratio, free-labelled-progesterone : bound-labelled-progesterone (F/B) against the amount of unlabelled progesterone gives an approximate straight line in the range

0-500pg progesterone. This is perferable because the progesterone in the unknown sample can be then determined by calculation rather than extrapolation.

Cross-reactivity of antiprogesterone antiserum

Standard solutions of many steroids which may have cross-reacted with the antiprogesterone antiserum were prepared in ethanol. From each, 500pg of steroid was compared with the standard curve for progesterone over the range 0-500pg. Table 2.IV lists the steroids tested and their cross-reactivity.

Testosterone RIA

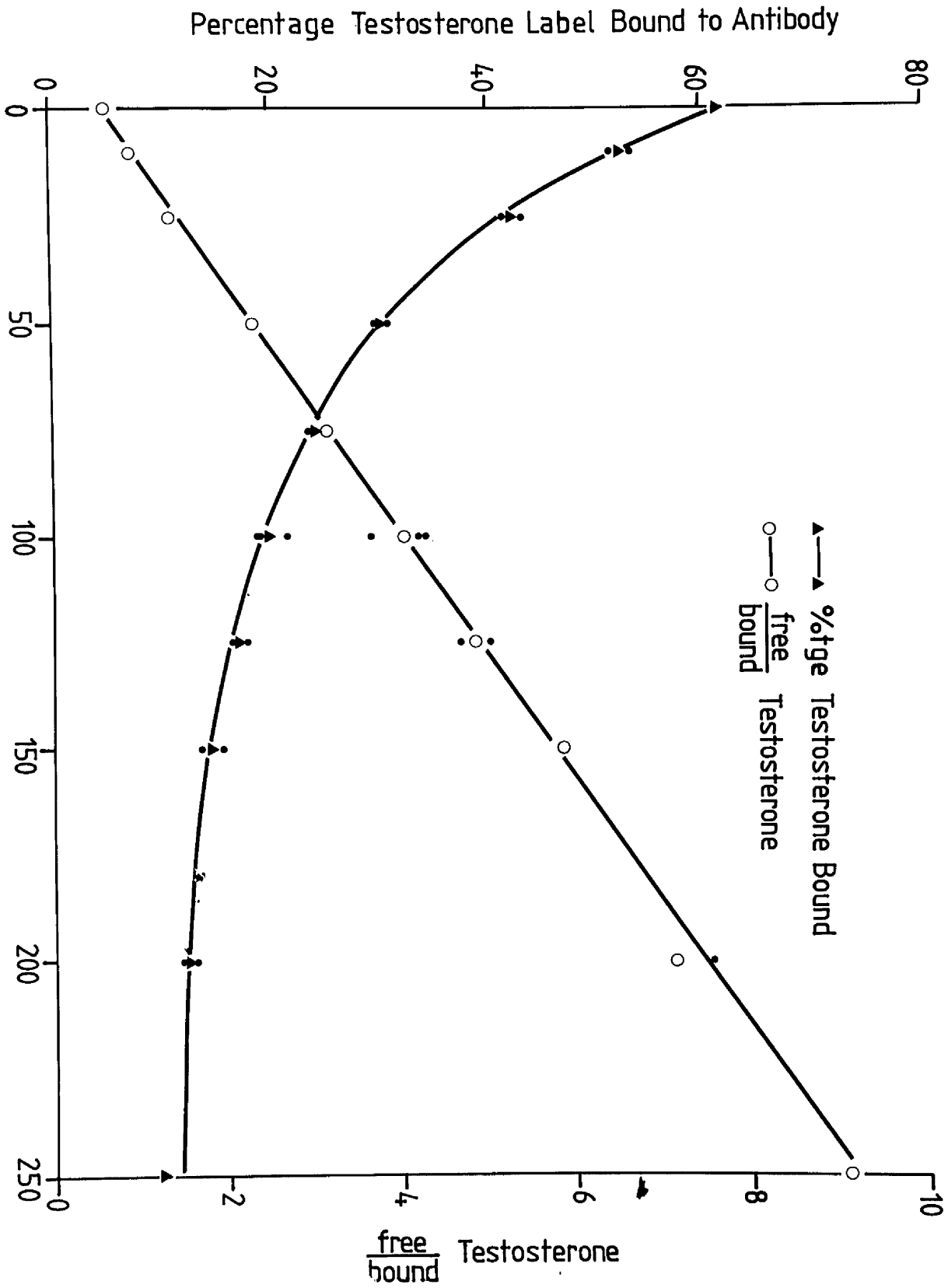
The testosterone RIA method used is similar to that described by Collins et al. (1972). Unlike the progesterone RIA which was developed for the present thesis, the RIA method for testosterone had already been established by other members of our department and optimum parameters had already been worked out for our laboratory conditions. The procedure is given in Appendix V.

The testosterone antibody binds testosterone more strongly than the antiprogesterone serum binds progesterone, therefore, there is less stripping of the testosterone by the dextran coated charcoal. For this reason, 20 minutes is allowed to elapse between addition of dextran charcoal and the start of the centrifugation (step vi) compared to the 5 minutes in the progesterone assay. As a result, testosterone assay is more reproducible than the progesterone assay. A typical standard curve is shown in Fig. 2.9.

Table 2.IV. Cross-reactivity of various steroids with anti-progesterone-11α-hemisuccinyl-BSA serum in the progesterone radioimmunoassay. 500 pg of each steroid was assayed and the "progesterone equivalent" determined from the progesterone standard curve.

Steroid (500 pg) *	Dextran charcoal separation			PEG separation		
	% tge bound	progesterone equivalent (pg)	cross reactivity (% tge)	% tge bound	progesterone equivalent (pg)	cross reactivity (% tge)
progesterone	9.2	500	100	25.5	500	100
11-ketoprogesterone	12.5	300	60	26.5	450	90
pregnanedione	20.7	105	21	40.7	173	35
5α-dihydrotestosterone	23.8	66	13	48	82	16
5α-androstanedione	24.5	57	11	46.5	96	19
5β-dihydrotestosterone	25	52	10	47.4	88	18
16α-hydroxy-progesterone	25	52	10			
oestrone	26.3	40	8	49.7	66	13
testosterone	27.7	32	6	45.4	108	22
20α-hydroxy-pregnenolone	27.7	32	6			
17α-hydroxy-20α-dihydroprogesterone	27.7	32	6			
17α-hydroxy-pregnenolone	28.8	25	5			
5α-pregnan-3, 20-dione	28.8	25	5			
16α-hydroxy-testosterone	30	18	4			
20β-dihydro-progesterone	30	18	4	57.2	22	4
androstenedione	30.5	15	3	46.5	96	19
7α-hydroxytestosterone	31	12	2.5			
17α, 20α-dihydroxy-pregnenolone	31.5	9	2			
androsterone	31.6	8	2	59.5	12	2
DHA	30.8	12	2	51	54	11
17α-hydroxy-progesterone	31.6	8	2	51.1	52	10
pregnenolone	31.5	9	2	51.3	50	10
5α-pregnan-3β, 20α-diol	33	5	1			
5α-pregnanolone	37.5	0	0	53.2	40	8
5β-pregnanolone	34.8	0	0	54.5	35	7
20α-dihydro-progesterone	37.5	0	0	56.8	25	5

Fig. 2.9. Standard curve for testosterone RIA.
Each point is the mean of three readings, the individual values being represented by small dots.


Percentage Testosterone Label Bound to Antibody
0
20
40
60
80
0
50
100
150
200
250
Standard Testosterone Added (pg)
%tge Testosterone Bound
free/bound Testosterone
0
2
4
6
8
10
free/bound Testosterone

As before, the ratio of free: bound tritiated testosterone (F/B) is plotted against unlabelled testosterone added to give an approximate straight line from which the amount of testosterone in an unknown sample can be calculated. Steroids tested for cross-reactivity with the antitestosterone antiserum are shown in Table 2.V.

Tissue culture medium was collected daily from monolayer cultures of foetal rat testes, and each medium was assayed for both progesterone and testosterone using RIA. Those media with a high concentration of steroid, as detected by RIA, were extracted for HPLC. This gave qualitative and quantitative confirmation of the presence of progesterone and testosterone, and also gave an indication of other steroids produced endogenously by the cultured cells.

Table 2.V. Cross-reactivity of various steroids with anti-testosterone-3-BSA antibody.

Steroid *	Cross reaction (% tge)
testosterone	100
7α-hydroxytestosterone	250
5α-dihydrotestosterone	150
dehydroepiandrosterone (DHA)	2
3β,17β-androstandiol	2
androstenedione	1
16α-hydroxytestosterone	1
progesterone	0.1
17α-hydroxyprogesterone	0.4
17α-hydroxy-20α-dihydroprogesterone	0.1
20α-dihydroprogesterone	0.5
16α-hydroxyprogesterone	0.1

* Nomenclature of steroids is given in Appendix I

Data from M. S. El-Khatim, Ph.D. thesis. University of London, 1976.

SECTION I : EXPERIMENTAL STUDIES

CHAPTER 3

ORGAN CULTURE OF FOETAL GENITAL TRACTS

RESULTS AND DISCUSSION

CHAPTER 3

RESULTS AND DISCUSSION

ORGAN CULTURE OF GENITAL TRACTS AND GONADS

A. RESULTS

Forty-six pregnant Wistar rats yielding 479 foetal rats (mean:- 10 foetuses per pregnant rat) were used in this survey. These pregnant rats were supposed to be at 14½ days gestation, but it became clear that many of the rats were at a different gestational age. Therefore, gestational age had to be assessed by the stage of development of the foetal gonads and genital ducts as shown in Table 3.I. A rat was acceptable as being at 14½ days gestation if the following criteria for the foetuses were met:-

(i) gonadal differentiation: The testes were just noticeably larger than the ovaries of female sibs under the dissecting microscope, and had a well marked surface vascular network. Histologically, at this time, there was little difference between the ovary and testis, the testicular tubules (primary sex cords) being poorly developed.

(ii) Wolffian duct: Histological sections showed fully developed Wolffian ducts in both sexes with no evidence of regression.

(iii) Mullerian duct: In both sexes, early anterior development of the Mullerian ducts was seen histologically but there was no evidence of canalisation and no regression had occurred.

Table 3.I. Development of rat foetal gonads and genital ducts at different gestational ages.

Gestational age	Foetal testis	Foetal ovary	Wolffian duct	Mullerian duct
$11\frac{1}{2}$ days	gonad not identified as a separate structure		fully developed	not present
$12\frac{1}{2}$ days	indifferent gonad just developed		fully developed	not present
$13\frac{1}{2}$ days	indifferent gonad		fully developed	very early anterior development
$14\frac{1}{2}$ days	just recognisable under dissecting microscope	indifferent: smaller than testis	fully developed	present anteriorly but not canalised
$15\frac{1}{2}$ days	well developed primary cords seen histologically	indifferent: smaller than testis	fully developed	almost completely developed almost reaching urogenital sinus
$16\frac{1}{2}$ days	well developed primary cords seen histologically	indifferent: smaller than testis	fully developed	posterior development complete: anterior regression in males
$17\frac{1}{2}$ days	well developed primary cords seen histologically	smaller than testis: ovarian tissue recognised by meiotic figures in secondary cords	fully developed: anterior regression in some females	regressed anteriorly in males: no regression in females

Twenty-three pregnant rats containing 230 foetuses were accepted as being at 14½ days gestation and of these, 48 foetal genital tracts were fixed for histology without culture to act as controls, and 153 were cultured for 3 days. The number of rats at different gestational ages is shown in Table 3.II.

1. Normal embryological development in vitro

a) Mullerian duct development in vitro

Mullerian duct development in vitro was observed in 129 cultures of genital tracts. In 29 cases, genital tracts were removed from foetal rats of 11½ to 13½ days gestation, at which time Mullerian duct development had not commenced, but after 3-4 days in culture they were detected histologically. A further 100 genital tracts were removed from older rat foetuses at a time when Mullerian duct development had just commenced: after 3 days in culture, their development had been completed in vitro (Fig. 3.1).

b) Mullerian duct regression in vitro

Male genital tracts with attached testes were explanted from 13 foetal rats of gestational age 14½ days (X9), 15½ days (X1) or 17½ days (X3) and maintained in organ culture for 2-4 days. At the end of this culture period, anterior Mullerian duct regression had occurred in vitro in the presence of the attached

Table 3.II. Gestational age of foetal rats whose genital tracts were used for organ culture.

Gestational age (days)	Number of pregnant mothers	Total number of foetal rats	Controls fixed without culture	Number of genital tracts cultured	Number of genital tracts not examined
$11\frac{1}{2}$	1	13	1	6	6
$12\frac{1}{2}$	2	24	3	21	0
$13\frac{1}{2}$	9	109	20	67	22
$14\frac{1}{2}$	23	230	48	153	29
$15\frac{1}{2}$	4	39	5	29	5
$16\frac{1}{2}$	1	9	2	7	0
$17\frac{1}{2}$	6	55	10	22	23
Totals	46	479	89	305	85

Fig. 3.1. Development of the foetal rat Mullerian duct *in vitro*.

Fig. 3.1a. Genital tract removed from a 14½ days gestation female foetal rat and fixed immediately in Bouin's fixative. This section, from the posterior portion of the genital tract, shows only one genital duct - the Wolffian duct. The Mullerian duct has not yet reached this level.

(H & E X 570)

Fig. 3.1b. Genital tract from a female litter-mate of above, removed at 14½ days gestation and maintained in organ culture for 3 days. Both genital ducts are now present, the Mullerian duct having developed *in vitro*.

(H & E X 570)

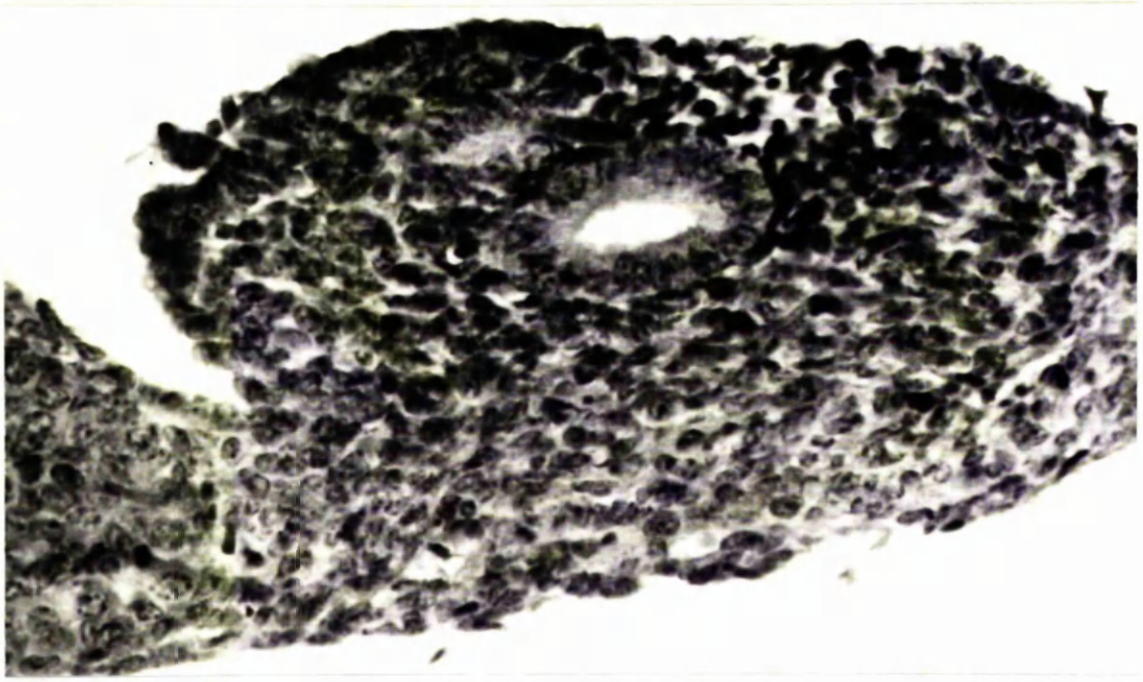


Fig. 3.1a.

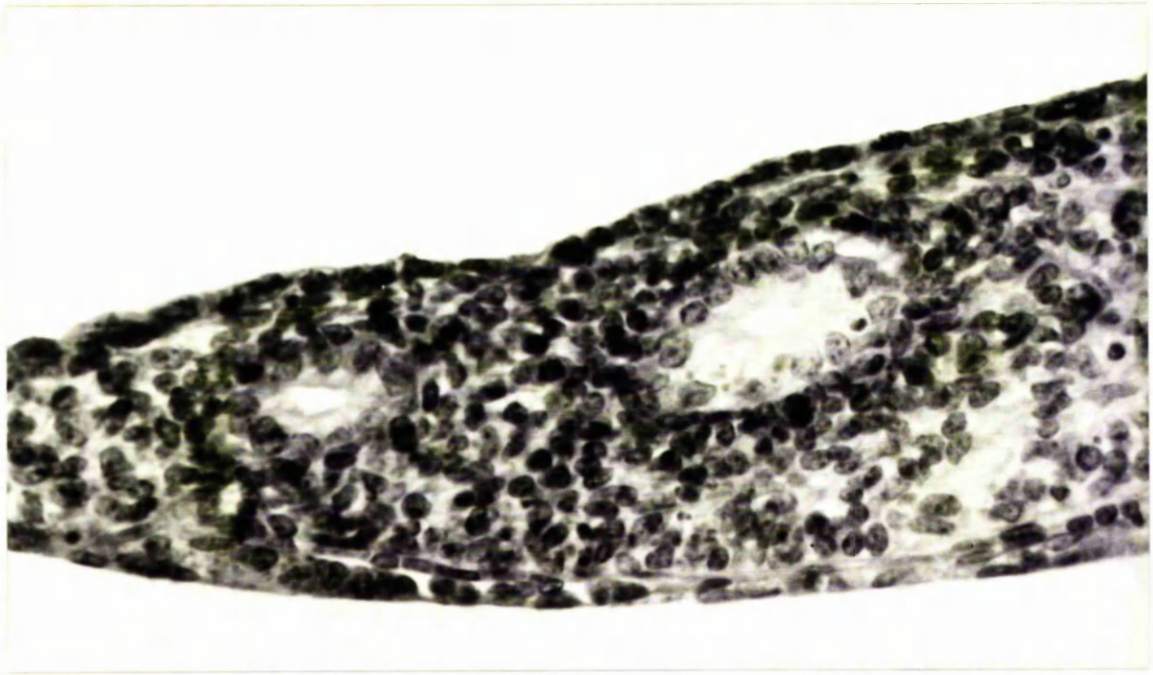


Fig. 3.1b.

testis whereas in 40 similar male genital tracts, removal of the testes prior to culture prevented *in vitro* Mullerian duct regression. Female genital tracts cultured with or without, attached indifferent gonads did not show Mullerian duct regression. Regression of the Mullerian duct *in vitro* only occurred if a foetal testis was adjacent to the genital tract, and if the gestational age at the start of the culture plus the number of days in culture equalled, or exceeded, 17½ days (Fig. 3.2).

In two instances, male genital tracts were unilaterally castrated before culture. After 3 days *in vitro* Mullerian duct regression was evident in the genital tract in contact with the single testis, but not in the contralateral genital tract which had been castrated (Fig. 3.3).

c) Wolffian duct development *in vitro*

The Wolffian ducts were fully developed at the start of the culture in every case, even in the youngest (11½ days gestation) foetuses dissected. Therefore, no further development was detected *in vitro*.

d) Wolffian duct regression *in vitro*

In the absence of testes (female, or castrated male genital tracts) Wolffian duct regression *in vitro* occurred if the gestational age at the start of the culture plus the number of days in culture exceeded 17½ days. This was observed in 59 cultures, *viz*. 10 whole female genital tracts, 29 castrated female genital tracts and 20 castrated male genital tracts (Fig. 3.4). The presence of a testis adjacent to the genital tract in culture

Fig. 3.2. Regression of the foetal rat Mullerian duct *in vitro* in the presence of co-cultured rat foetal testes.

Fig. 3.2a. Genital tract removed from a 14½ days gestation foetal rat and fixed in Bouin's immediately. This section, from the middle portion of the genital tract, shows both genital ducts to be present. The Mullerian duct is more lateral, towards the free border of the genital tract.

(H & E X 570)

Fig. 3.2b. This is a similar genital tract explanted from a 14½ days gestation male foetal rat and maintained for 3 days in organ culture with the foetal testis remaining attached. The Wolffian duct persists, but the Mullerian duct has regressed completely because of testicular MIF.

(H & E X 570)

Fig. 3.2a.

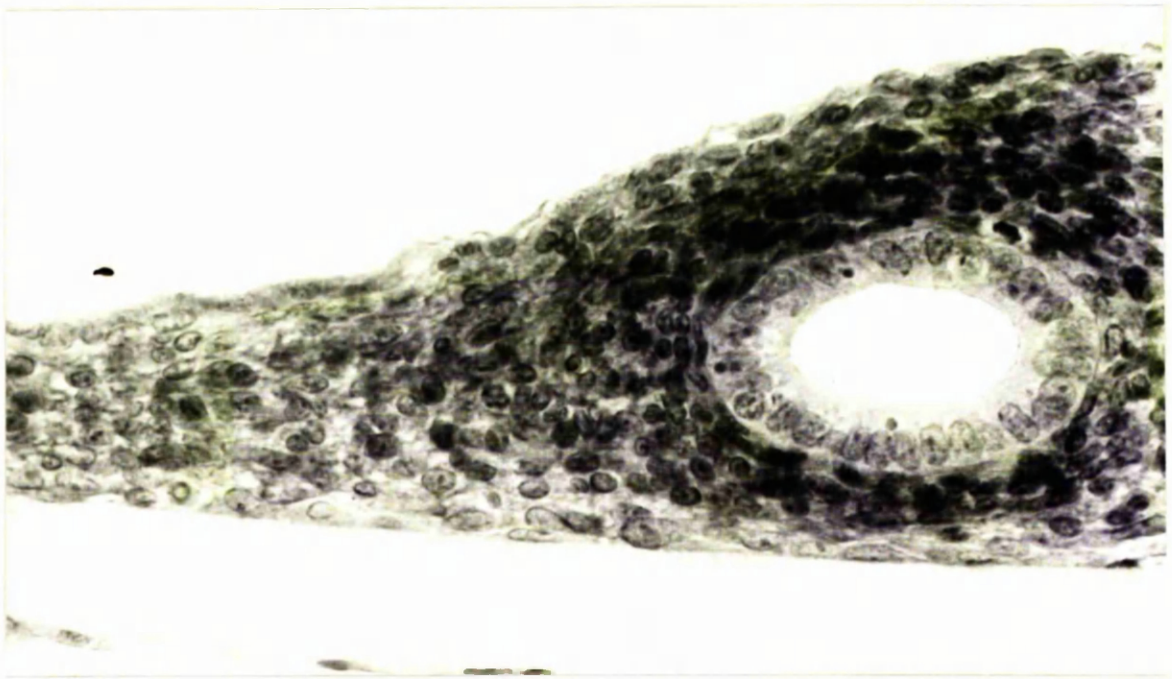


Fig. 3.2b.

Fig. 3.2. Regression of the foetal rat Mullerian duct *in vitro* in the presence of co-cultured rat foetal testes.

Fig. 3.2c. Lower magnification of Fig. 3.2b showing the adjacent foetal testis. The Wolffian duct is present at the left hand edge of the cultured genital tract, and the testis is present at the right hand border. Mesonephric tubules are seen in the central portion of the tract.

(H & E X 200).

Fig. 3.3. Unilateral regression of the Mullerian duct *in vitro* after unilateral castration.

Fig. 3.3a. Genital tract explanted from a 14½ days gestation male foetal rat and maintained for 3 days in organ culture. This side was not castrated, the testis being in contact with the genital tract throughout the culture period. The Wolffian duct (left hand duct) persists and is viable, but the Mullerian duct has regressed.

(H & E X 570)

Fig. 3.3b. This contralateral genital tract was castrated before culture, the testis being removed before explantation. After 3 days in organ culture the absence of an adjacent testis allows persistence of the Mullerian duct (right hand duct) but the Wolffian duct is regressing.

(H & E X 570)

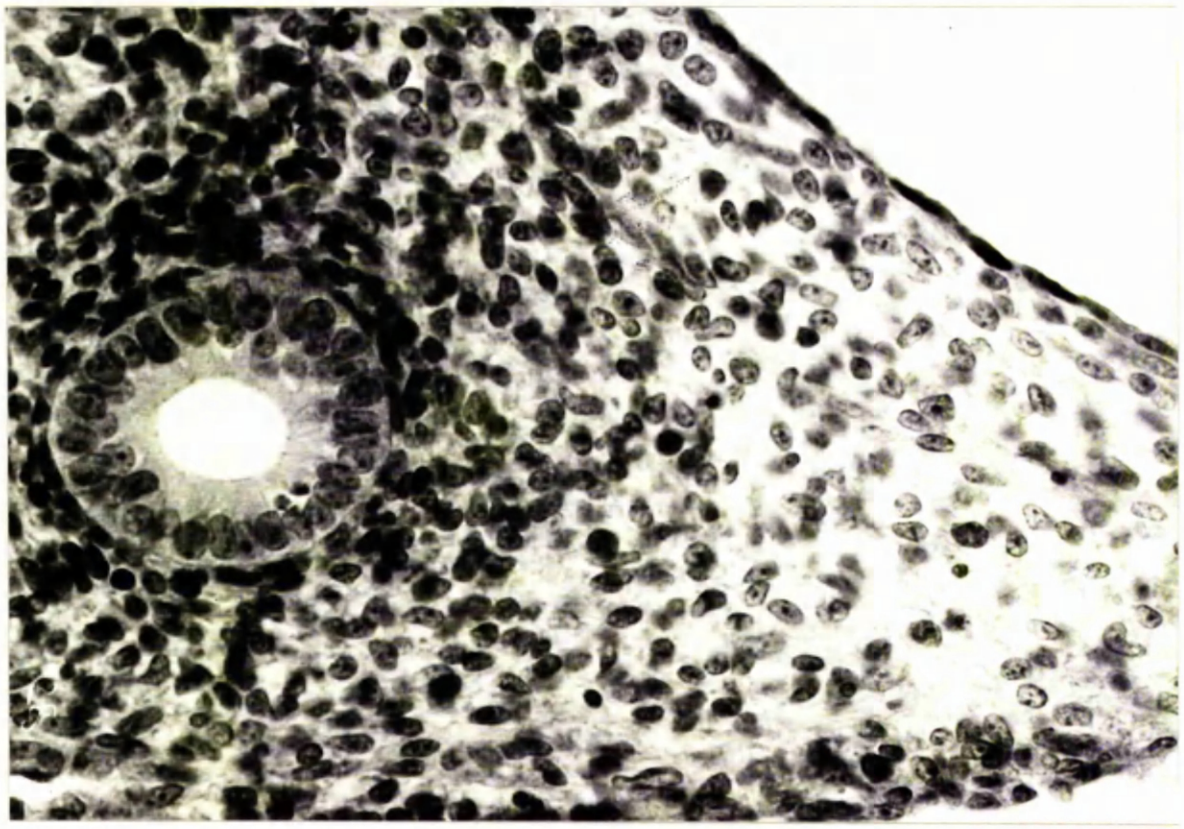


Fig. 3.3a

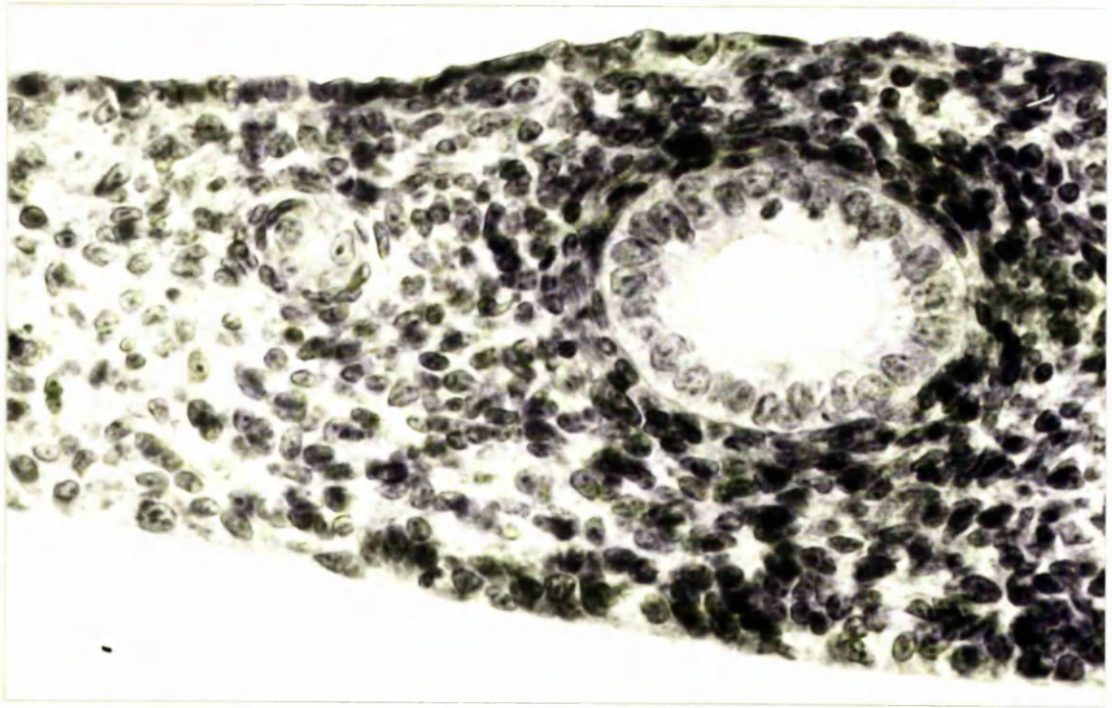


Fig. 3.3b

Fig. 3.4. Regression of the Wolffian duct *in vitro* in the absence of a foetal testis.

Fig. 3.4a. Genital tract removed from a 14½ days gestation foetal rat and fixed immediately in Bouin's. This section shows the presence of the Wolffian duct but the Mullerian duct has not yet fully developed. (H & E X 570)

Fig. 3.4b. This foetal rat genital tract was explanted from a female sib of the above foetus and maintained in organ culture for 3 days. Although the ovary is intact, the absence of a testis has resulted in persistence of the Mullerian duct, but the Wolffian duct (left hand duct) is regressing. (H & E X 570)

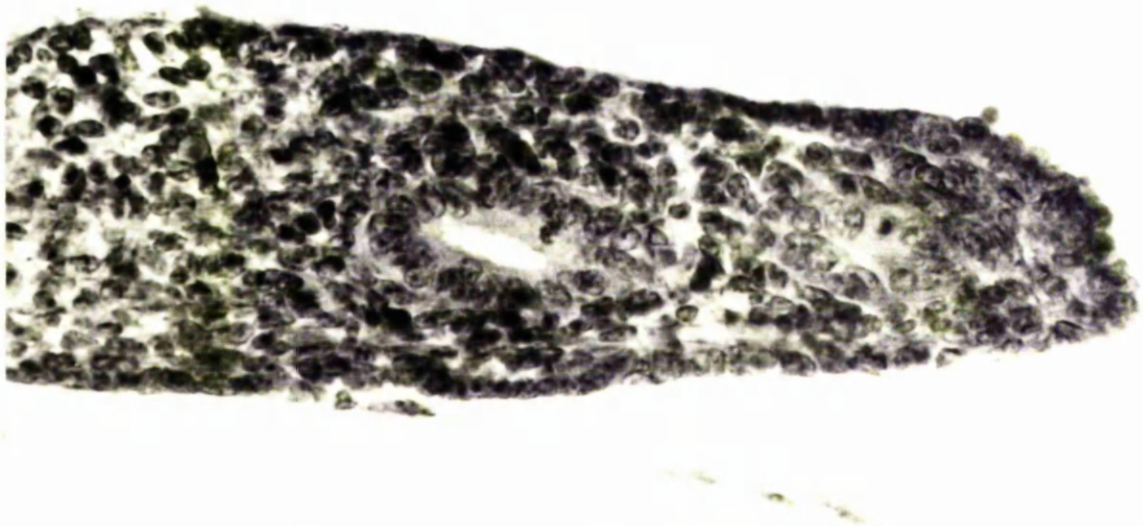


Fig. 3.4a

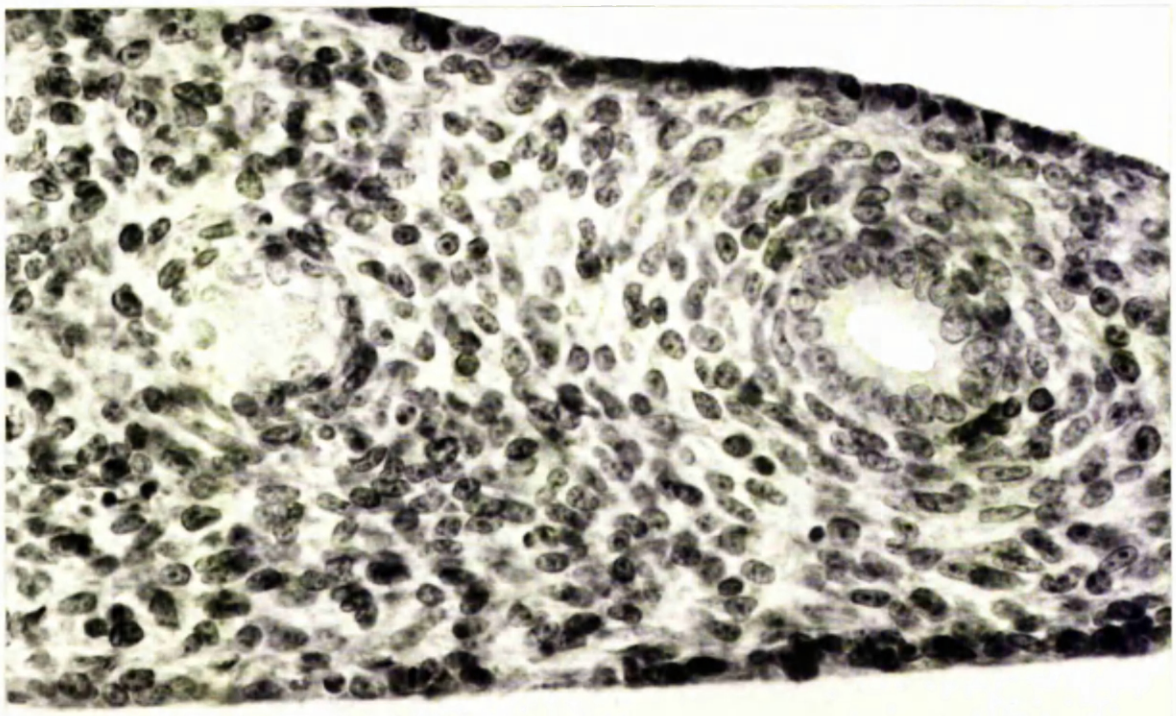


Fig. 3.4b

prevented Wolffian duct regression *in vitro*.

e) Gonadal differentiation *in vitro*

One pregnant rat was killed at 11½ days gestation. The genital tracts of the foetuses could be identified, but there was no evidence of gonadal tissue under the dissecting microscope, nor histologically. Six of these genital tracts were cultured for 6 days after which time histologically identifiable indifferent gonads had developed *in vitro* in two.

In 11 cultures, genital tracts from 13½ day foetuses had indifferent gonads at the start of the culture , but after 3 days *in vitro*, recognisable testes with primary testicular sex cords had developed. In another 20 cultures, indifferent gonads at the start developed into ovaries identified by the presence of meiotic figures in the germ cells of the secondary sex cords in the cortex (Fig. 3.5).

The above series of experiments confirm that normal development of gonads, and normal development and regression of genital ducts can be reproduced *in vitro* in organ culture, and the time scale *in vitro* closely approximates to the time scale *in vivo*. They also confirm that the foetal testis is responsible for Mullerian duct regression and Wolffian duct stabilisation, and that the influence of the foetal testis is unilateral. Further confirmation of this can be achieved by transplanting foetal testes, as in the following experiments:-

a) Transplanted foetal rat testes

Castrated female genital tracts explanted from 2 foetal rats of 14½ days gestation were cultured with foetal testes

Fig. 3.5. Differentiation of rat foetal gonads *in vitro*.

Fig. 3.5a. Genital tract removed from a 13½ days gestation foetal rat and fixed immediately in Bouin's. The gonad could not be sexed under the dissecting microscope. This histological section shows the ill-defined primary sex cords of the indifferent gonad. At this stage, the forerunner of the testis is identical to that of the ovary. (H & E X 570)

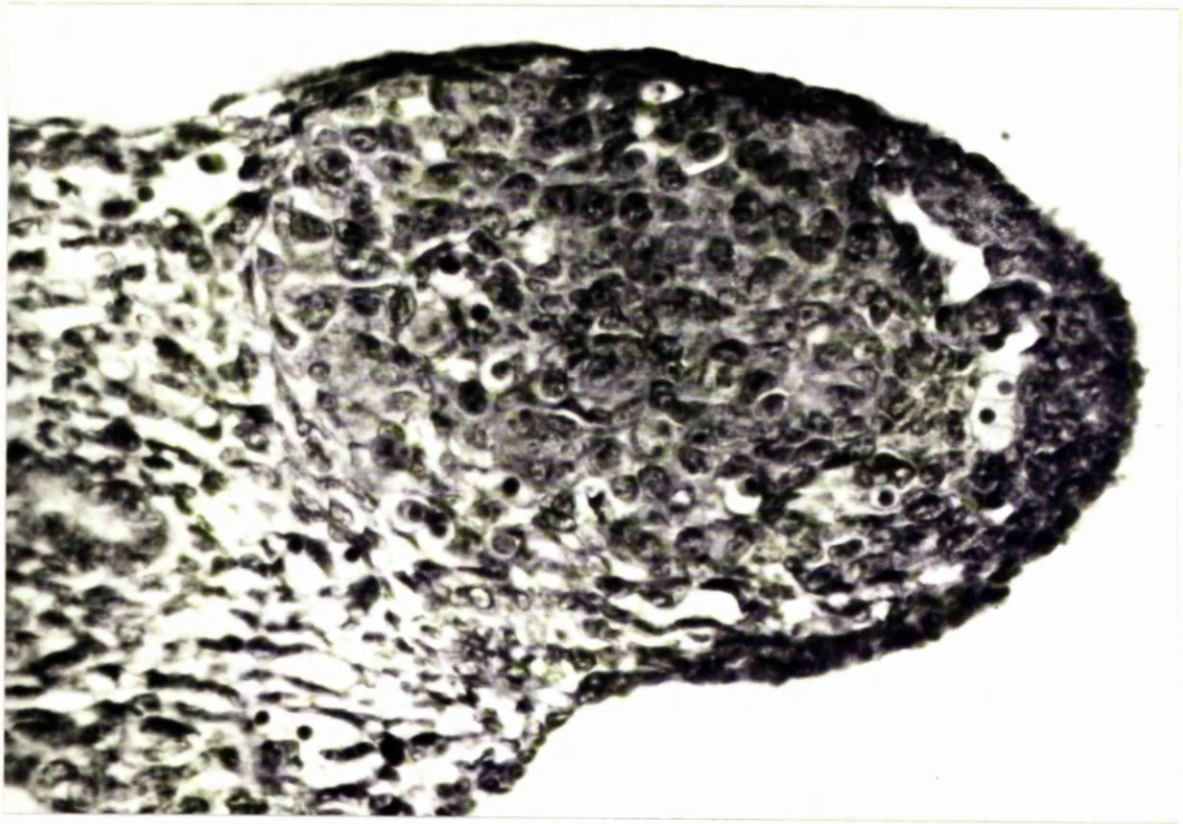


Fig. 3.5a

Fig. 3.5b. Genital tract explanted from a 13½ days gestation foetal rat (sib of Fig. 3.5a.) and maintained in organ culture for 4 days. The primary sex cords have expanded and differentiated into primitive seminiferous tubules with germ cells and Sertoli cells. This is a recognisable foetal testis. (H & E X 570)

Fig. 3.5c. Genital tract explanted from a 13½ days gestation foetal rat (sib of Fig. 3.5a.) and maintained in organ culture for 4 days. The primary sex cords of the gonadal medulla have regressed, and the germ cells are recognised by their meiotic activity causing expansion of the cortex of the ovary. (H & E X 570)

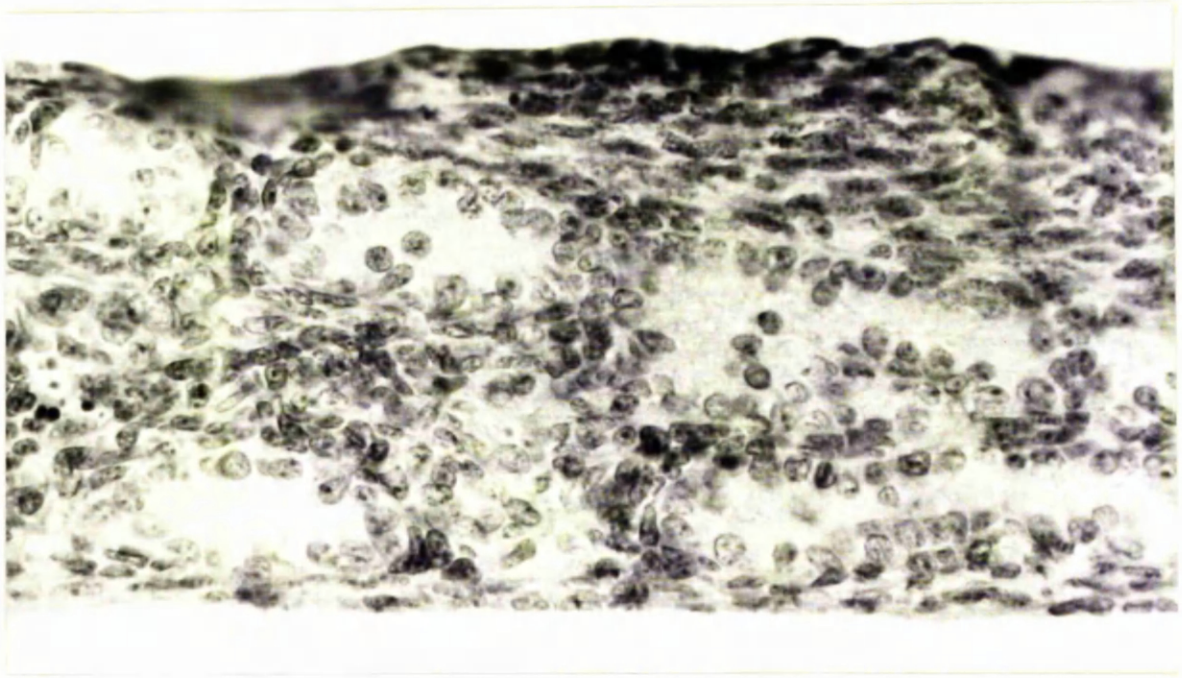


Fig. 3.5b

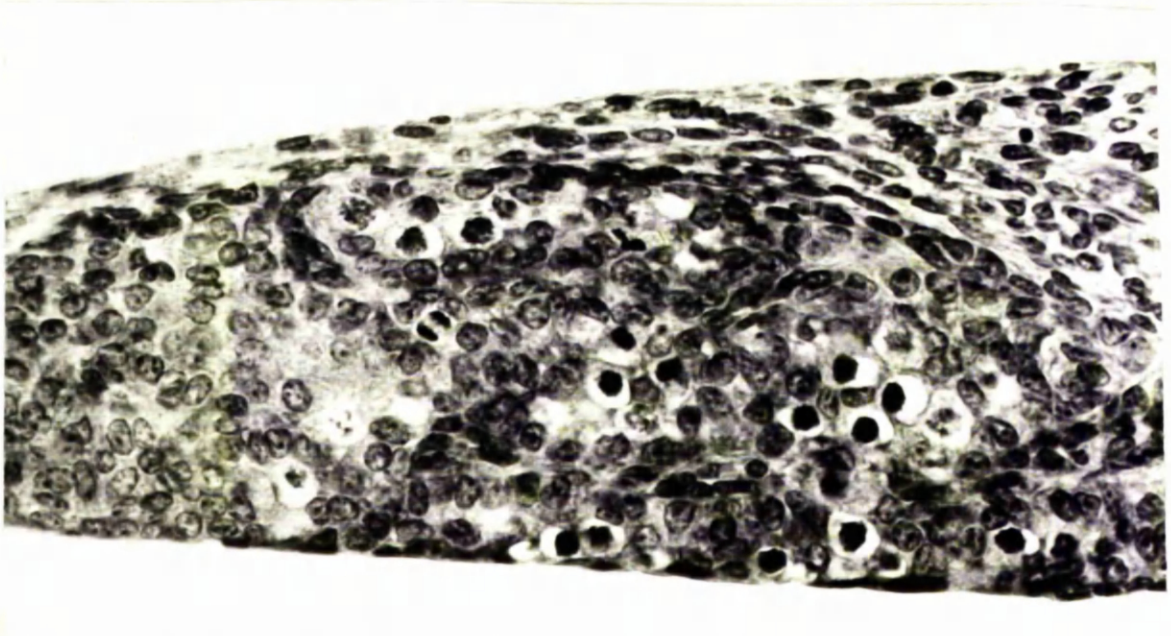


Fig. 3.5c

removed from male litter mates. The testes were placed adjacent to the genital tracts from whence the female gonad had been removed. After 3 days in culture, the Mullerian ducts had regressed and the Wolffian ducts were present, whereas castrated female genital tracts without transplanted testes showed Wolffian duct regression and Mullerian duct persistence after the same culture period.

b) Transplanted foetal human testes

Testes from 7 human foetuses ranging from 12 to 24 weeks gestation, were cut into small portions - approximately 1mm diameter - and maintained in organ culture. These were placed adjacent to castrated rat foetal genital tracts explanted from 17 rat foetuses ranging from 12½ to 16½ days gestation. The rat foetal genital tracts and adjacent human foetal testes were cultured for a few days, after which time the human foetal testes were shown to have caused stabilisation of the Wolffian ducts, and inhibition of the Mullerian ducts in 11 different cultures. This shows that in the absence of rat foetal testes, normal genital duct stabilisation and regression in the rat foetal genital tract can be affected by human foetal testes. Therefore, the humoral factors involved are not species specific.

c) Transplanted pregnant rat ovary

Portions of pregnant rat ovary were cultured adjacent to 19 foetal rat genital tracts on the assumption that the pregnant ovary was a source of progesterone. After 3 days in organ culture, all the pregnant rat ovaries were completely necrotic and there was no evidence of Mullerian duct inhibition. The presence of HCG in the culture medium had no effect on the genital ducts and did

not prevent degeneration and necrosis of the pregnant rat ovary.

3. Effect of exogenous steroid hormones on genital ducts in vitro

The steroid hormones testosterone and progesterone were added to some culture media. Concentrations of up to 10^{-4}M were used in order to observe a definite effect, although this concentration is pharmacological, and grossly in excess of the normal physiological concentration in the circulation. Testosterone and progesterone are not soluble in aqueous solution at such high concentrations, but are taken up by the proteins of the foetal calf serum in the culture media. The steroids were dissolved in analytical grade ethanol and added to the culture media.

a) Effect of ethanol in culture medium

Because steroid hormones were added to the culture medium in ethanol, a similar concentration of ethanol was used in the control cultures. Culture media containing 1% analytical grade ethanol caused non-specific cytotoxic effects in 18 genital tracts maintained in organ culture for 3 days. However, in another 8 genital tracts this concentration of ethanol had no apparent deleterious effects. Because 1% ethanol may be toxic, a maximum concentration of 0.2% ethanol, which had no demonstrable toxic effects, was used in subsequent cultures.

b) Non-toxicity of testosterone

Testosterone at a concentration of 10^{-4}M was used in 37 cultures of foetal genital tracts.

No toxic effect attributable to testosterone was observed indicating that a steroid at 10^{-4}M concentration need not be toxic. Testosterone did not affect the Mullerian ducts, nor gonadal differentiation *in vitro*. The presence of testosterone in the culture medium was shown to stabilise the Wolffian duct in the absence of a foetal testis (Fig. 3.6).

c) Effect of progesterone in culture medium

Progesterone was included in the culture medium on 75 occasions, the final concentration being 10^{-5}M (X5), $5X10^{-5}$M (X17) or 10^{-4}M (X53).

(i) Effect of progesterone on the Mullerian duct (Table 3.III): 10^{-5}M progesterone had no noticeable effect on the Mullerian duct in organ culture, but 5×10^{-5}M on 6 occasions, and 10^{-4}M on 21 occasions, had specific Mullerian duct inhibitory effects without inhibition of the Wolffian duct. These Mullerian inhibitory effects were seen in cultures of genital tracts explanted from 11½ days (X3), 13½ days (X9), 14½ days (X12), 15½ days (X1) and 16½ days (X2) foetuses. In the younger tracts, progesterone prevented the development of the Mullerian ducts *in vitro* and in the older tracts the pre-existing Mullerian ducts regressed *in vitro* (Fig. 3.7). However, in some cultures progesterone failed to inhibit the Mullerian ducts (Table 3.III), and in other cultures, generalised cytotoxicity of the cultured tissue was observed indicating a non-specific deleterious effect of progesterone.

Fig. 3.6. Stabilisation of the Wolffian duct *in vitro* by exogenous testosterone in the culture medium.

Fig. 3.6a. Genital tract castrated and explanted from a 14½ days gestation foetal rat and maintained for 3 days in organ culture in the presence of 0.1% ethanol, but no exogenous steroid hormone. The Mullerian duct (right hand duct) persists and remains viable, but the Wolffian duct is regressing because of lack of stabilising androgens.

(H & E X 570)

Fig. 3.6b. A similar castrated foetal rat genital tract explanted at 14½ days gestation. This tract was maintained in organ culture for 3 days in the presence of 0.1% ethanol and 10^{-4}M testosterone. The Mullerian duct is unaffected by the testosterone and remains viable. However, the Wolffian duct (left hand duct) has been stabilised and testosterone has prevented its regression *in vitro*.

(H & E X 570)

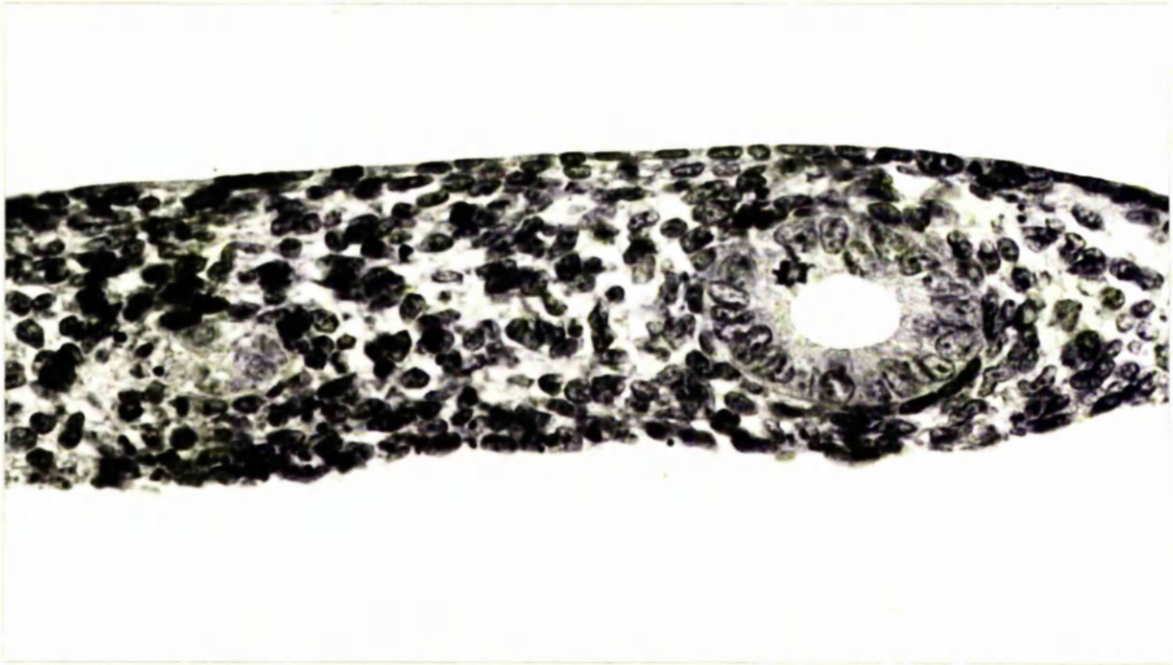


Fig. 3.6a

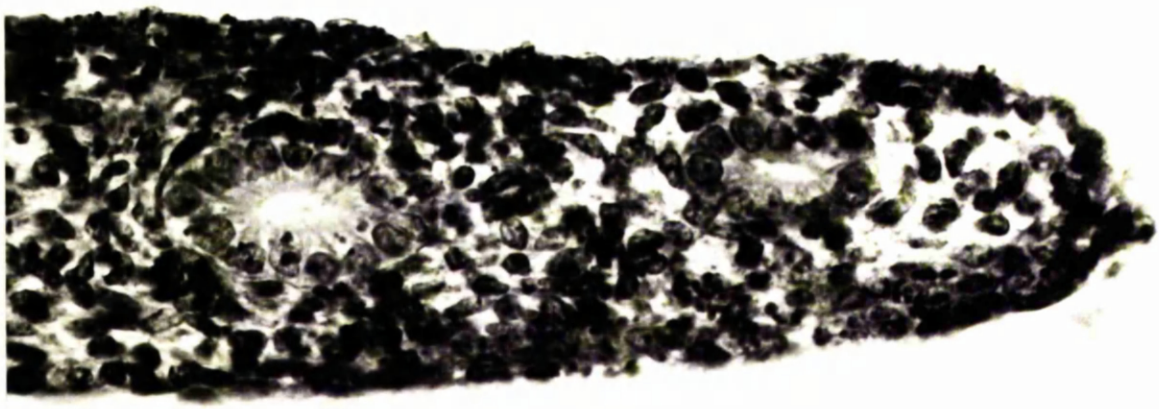


Fig. 3.6b

Table 3. III. Effect of progesterone on the Mullerian ducts in rat foetal testes in vitro.

Concentration of progesterone in culture medium	Effect of progesterone on genital tracts: Number of cultures showing	
	Mullerian duct inhibition	Lack of Mullerian duct inhibition
10^{-5} M	0	5
5×10^{-5} M	6	10
10^{-4} M	21	2

Fig. 3.7. Inhibitory effect of exogenous progesterone in the culture medium on the foetal Mullerian duct in organ culture.

Fig. 3.7a. Genital tract removed from a 15½ days gestation foetal rat, and fixed immediately in Bouin's. Sections from the anterior and posterior portions of the genital tract show that the Wolffian duct is fully developed and has not regressed. The Mullerian duct is present posteriorly and has reached the urogenital sinus, but anterior regression has commenced. (H & E X 570)

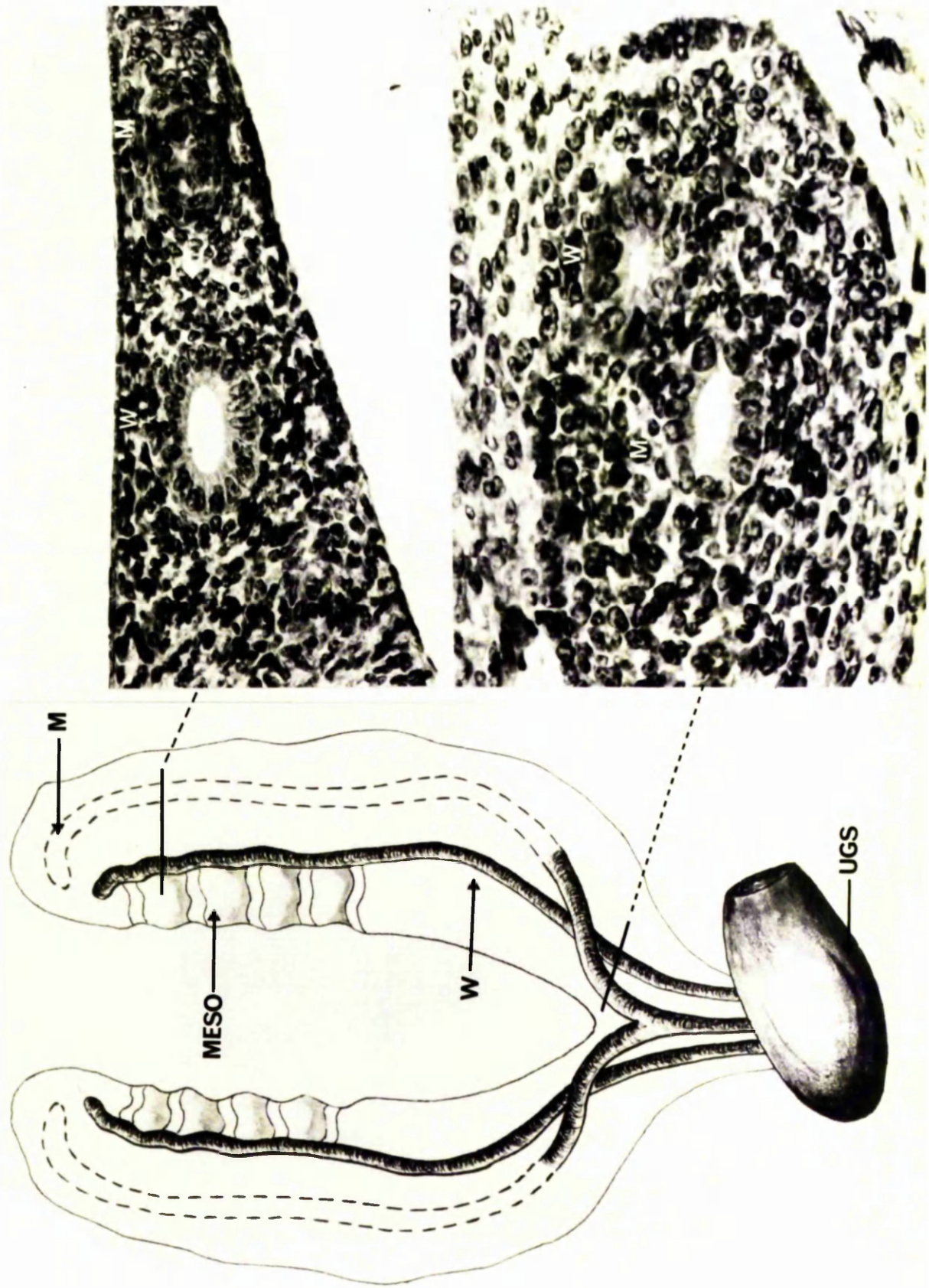


Fig. 3.7a.

Fig. 3.7b. This is a male genital tract and a sib of Fig. 3.7a. It was castrated and explanted at 15½ days gestation, and maintained in organ culture for 2 days in the presence of 0.1% ethanol but no added steroid hormone. The Wolffian duct remains intact, and castration has prevented further Mullerian duct regression, it still being present posteriorly. (H & E X 570)

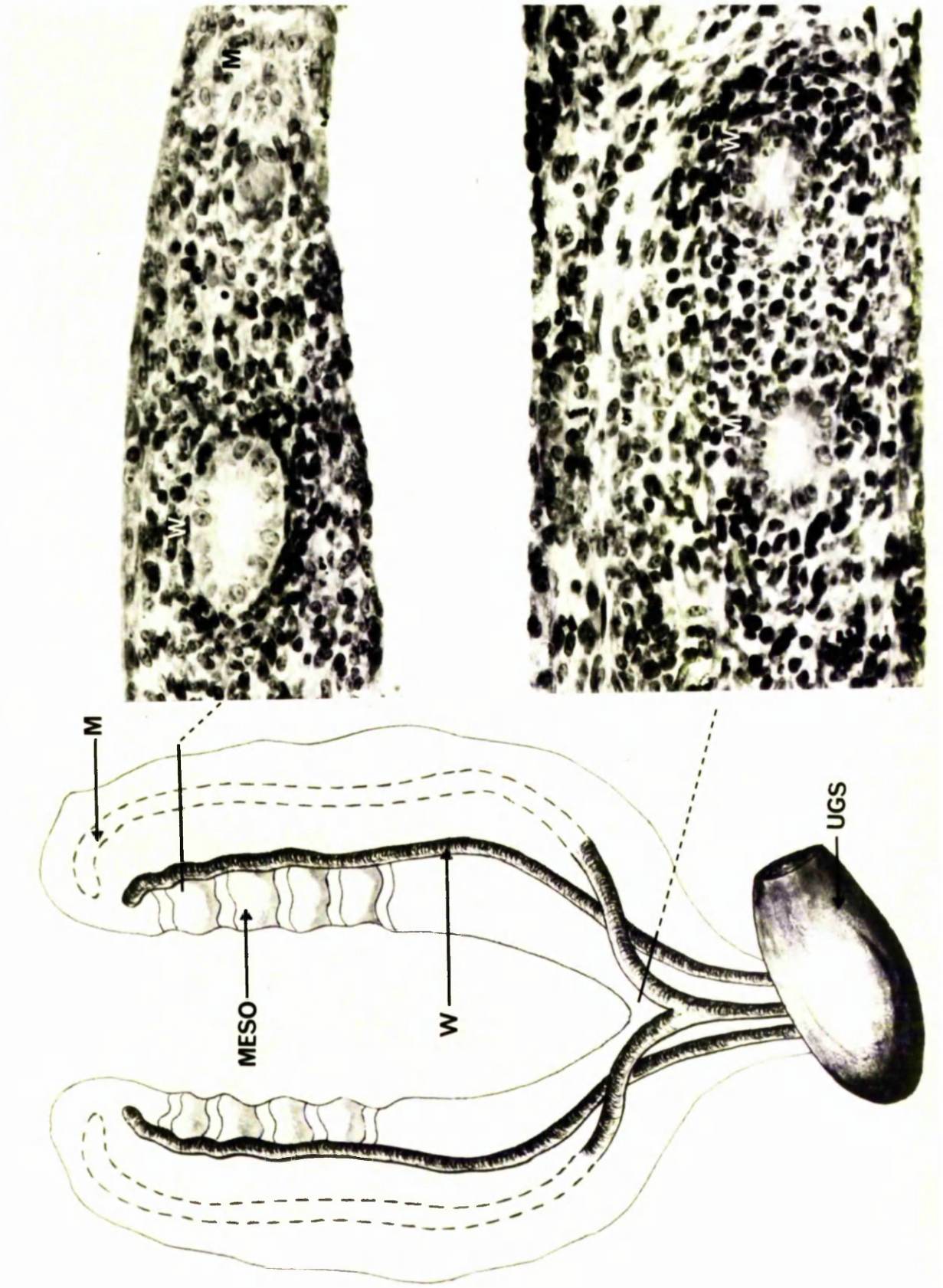


Fig. 3.7b.

Fig. 3.7c. This male genital tract, also a sib of Fig. 3.7a, was castrated and explanted into organ culture at 15½ days gestation, at the same time as Fig. 3.7b. After 2 days in culture in the presence of 0.1% ethanol and 10^{-4}M progesterone, the Wolffian duct has not altered, but Mullerian duct regression is now complete. Progesterone has simulated the inhibitory effect of the foetal testis. (H & E X 570)

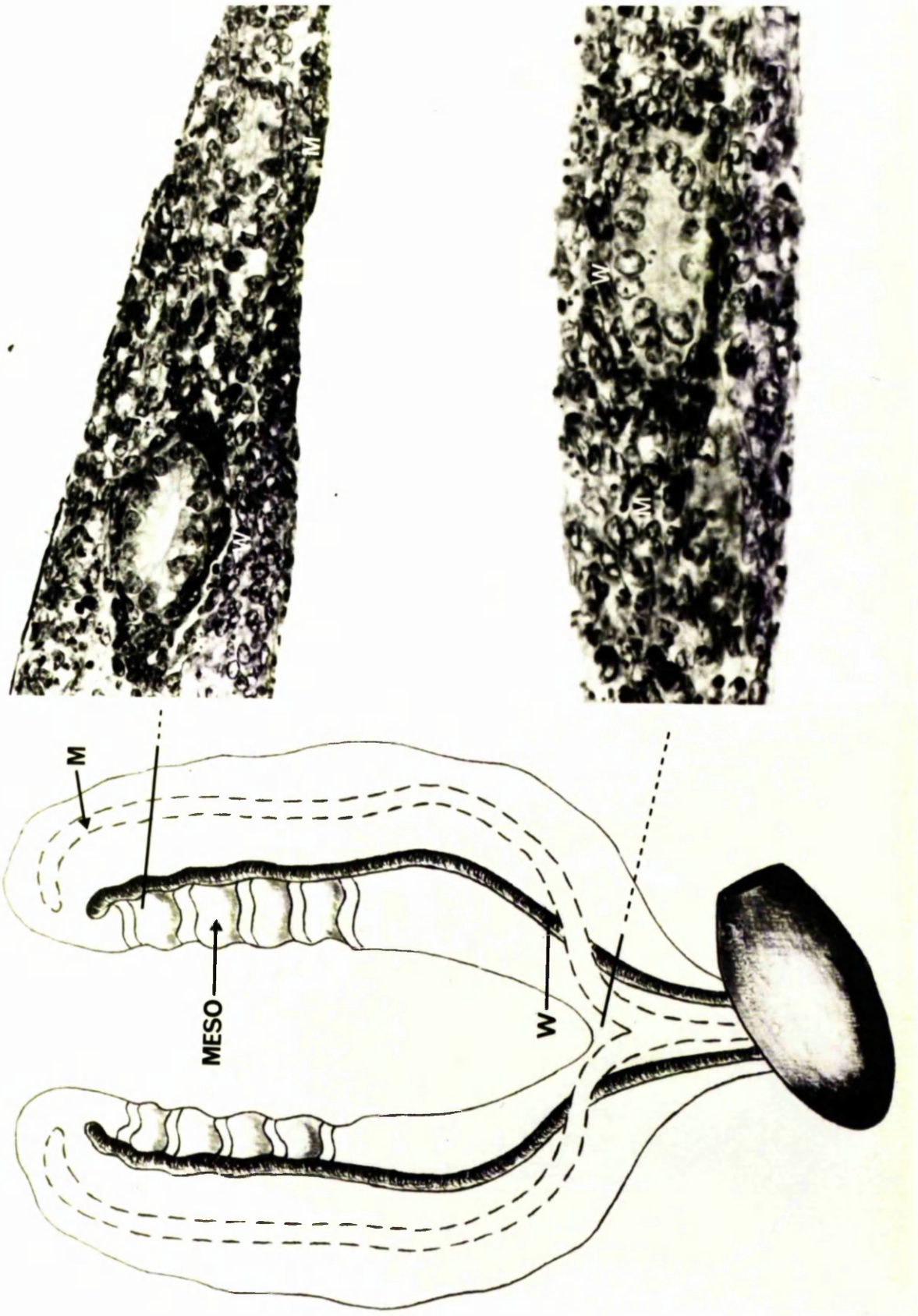


Fig. 3.7c.

(ii) Effect of progesterone on the Wolffian duct: In the cultures showing generalised cytotoxicity due to progesterone, the Wolffian ducts as well as the Mullerian ducts showed some regressive changes, but tended to be more resistant than the Mullerian ducts to the toxic effects of progesterone. Normally, progesterone did not inhibit the Wolffian ducts, and in 18 cultures the presence of progesterone in the culture medium caused Wolffian duct stabilisation (Fig. 3.8) compared to the control cultures which did not have added progesterone. Progesterone concentrations 10^{-5}M (X3), 5 X 10^{-5}M (X3) and 10^{-4}M (X12) showed Wolffian duct stabilisation activity.

Fig. 3.8. Stabilisation of the Wolffian duct *in vitro* by exogenous progesterone in the culture medium.

Fig. 3.8a. Genital tract castrated and explanted from a 14½ days gestation foetal rat and maintained for 3 days in organ culture in the presence of 0.1% ethanol, but no exogenous steroid hormone. Mullerian duct development has progressed normally to completion *in vitro* and it has not regressed. The Wolffian duct has almost completely regressed because of lack of stabilising androgens.

(H & E X 570)

Fig. 3.8b. A similar castrated foetal rat genital tract explanted at 14½ days gestation at the same time as its sib (above) and maintained for 3 days in organ culture in the presence of 0.1% ethanol and 10^{-4}M progesterone. In this case, the Mullerian duct has regressed and the Wolffian duct has been stabilised by the added progesterone.

(H & E X 570)

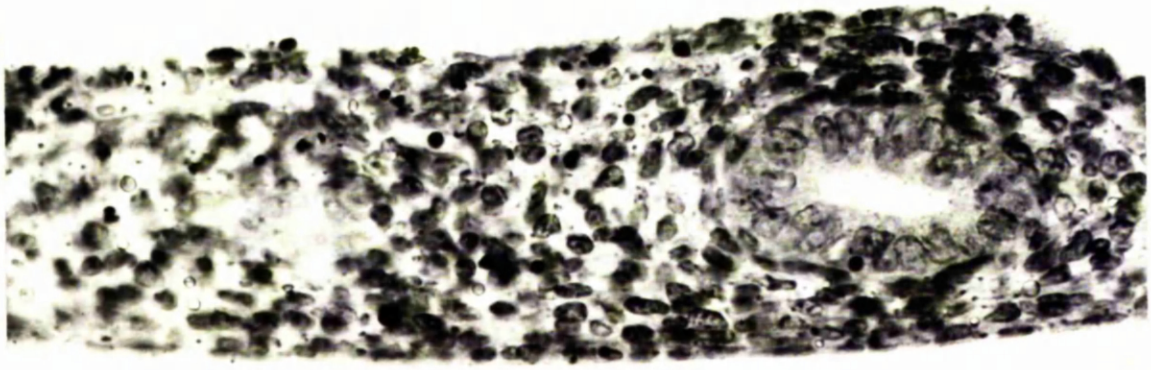


Fig. 3.8a

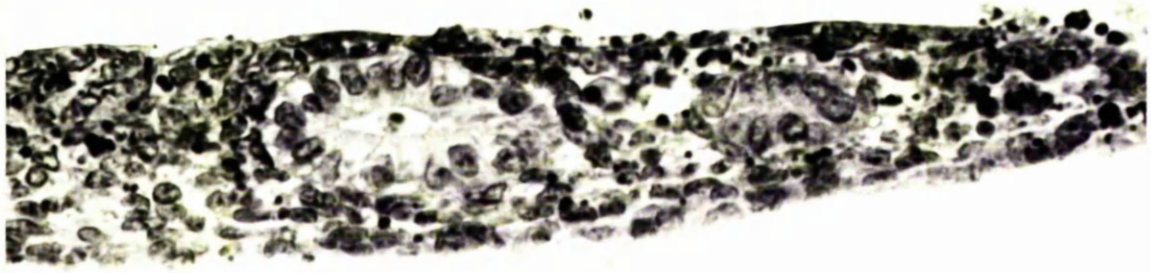


Fig. 3.8b

B. DISCUSSION

The organ culture of foetal rat genital tracts is a good model for studying the development of foetal gonads and genital ducts. Gonadal tissue may develop *in vitro* when there is no evidence of gonadal tissue at the start of the culture and explanted indifferent gonads may differentiate into testes or ovaries in culture (Fig. 3.5). This development and differentiation occurs in the absence of added hormonal factors.

The presence of foetal testis adjacent to a genital tract causes Mullerian duct inhibition and Wolffian duct stabilisation demonstrable at 17½ days gestation in the foetal rat *in vivo* or *in vitro* (Figs. 3.2&3.3). The foetal testis producing these effects may be autologous, homologous (from a sib) or heterologous (from a human foetus). These effects have been described by many workers (see introduction) and are verified by the experiments in the present series.

Testosterone causes Wolffian duct stabilisation in culture (Josso, 1970a; Price & Pannabecker, 1959) and *in vivo* (Elgar, 1966; Jost, 1953,1967). The present experiments clearly demonstrate that concentrations of testosterone up to 10^{-4}M are non-toxic to explanted foetal rat genital tracts and gonads in organ culture. However, progesterone at a concentration of 10^{-4}M may have non-specific cytotoxic effects in such cultures. The Mullerian duct is more sensitive to progesterone than the Wolffian duct, and in many cases progesterone results in Wolffian duct stabilisation and specific Mullerian duct inhibition.

The androgenicity of progesterone has been known for a long time (Green *et al*., 1939; Hill, 1937; Lamar,1940) which probably

explains its ability to stabilise the Wolffian duct. However, the direct stimulatory effect of progesterone on the Wolffian duct *in vivo* or *in vitro* has not been previously published, and this is the first report of its stabilising effect in organ culture.

An *in vitro* bioassay for androgens using rat ventral prostate in organ culture has been described (Zaaijer *et al.*, 1966). Using this system, it has been claimed that foetal testes in organ culture produce androgens (Ortiz *et al.*, 1966). However, in view of the known androgenic effect of progesterone on the prostate (Clausen, 1942; Green *et al.*, 1939) and the stimulatory effect of progesterone on the Wolffian duct described in this thesis, doubt must be cast on the conclusion that this bioassay system specifically detects secretion of conventional androgens by the foetal testis in culture. It is also possible that the bioassay was responding to progesterone secretion.

The stimulatory effect of progesterone on the Wolffian duct in organ culture can be explained by its androgenic activity, but does not necessarily imply that progesterone is produced by the foetal testis at the time of Wolffian stabilisation. Testosterone is produced at this time and is probably the active principle involved *in vivo*. However, the inhibitory effect of progesterone on the Mullerian duct cannot be as readily explained unless it is postulated that progesterone is involved in Mullerian inhibition *in vivo*.

Josso (Josso, 1972a, 1974b; Josso *et al.*,1975; Picard & Josso, 1976) has indicated that MIF is not a steroid in free form, but is probably a polypeptide or protein. However, it is also possible that MIF is a protein - steroid complex. The adult testis is

known to produce an androgen binding protein (ABP) which is synthesised by Sertoli cells under the influence of FSH, and which passes into the rete testis and epididymis but not the blood stream (Means et al., 1976). It is a carrier protein for testosterone and dihydrotestosterone, and is taken up by the epithelial cells of the epididymis being concentrated by the head of the epididymis. Only very small amounts reach the tail of the epididymis (Hanssen et al., 1973). Its exact function is unclear, but it is thought to stimulate spermatogenesis and spermatid maturation by maintaining a high concentration of androgen in the seminiferous tubules and epididymis (Hanssen et al.,1974.

It has been claimed that ABP is a specific marker for Sertoli cell function in adult rats (Weddington et al., 1975) and that MIF is a specific marker for Sertoli cell function in foetal rats (Tran et al., 1977). Tran et al. (1977)postulate that the two substances may be the same molecule. Naftolin (1976) also suggests that ABP might be MIF, but Ritzen (1976) has not been able to detect ABP in foetal testes. Bardin (1976) concludes that MIF is not ABP because MIF disappears after birth. However, ABP is only a carrier protein and the active principle is the androgen present in the complex. It may also be true that MIF is a carrier protein for a Mullerian inhibitory steroid, for example progesterone. If so, MIF may be closely related to - or identical with - ABP, and the lack of Mullerian inhibitory properties of ABP may be because the correct steroid has not been attached.

The binding of progesterone to ABP has not been described. If MIF is analogous to ABP, requiring a bound steroid for its action on the Mullerian duct, this would explain the localised

action of MIF because the protein-steroid complex would pass directly to the genital ducts without entering the foetal circulation.

The inhibitory effect of progesterone on the Mullerian duct in organ culture has not been previously described, and the possibility that progesterone is involved in Mullerian inhibition in vivo is a new postulate. The results of the experiments described in this section may possibly add credence to the postulate that progesterone is the active principle, in an MIF-steroid complex required for Mullerian duct inhibition. further substantiation of this postulate may be obtained if it can be shown that foetal testes produce and release progesterone at the time of Mullerian inhibition. Experiments using rat foetal testes in monolayer culture were performed in an attempt to demonstrate progesterone secretion by foetal testes, and these experiments are described in the next chapter.

SECTION I : EXPERIMENTAL STUDIES

CHAPTER 4

MONOLAYER CULTURE OF FOETAL TESTES

RESULTS AND DISCUSSION

CHAPTER 4

RESULTS AND DISCUSSION

MONOLAYER CULTURE OF FOETAL TESTES

A. RESULTS

Of eighteen monolayer cultures of foetal rat testes which were prepared, twelve cultures were able to be maintained for at least 20 days, these being prepared from foetal rats of 14½ (X1), 15½ (X2), 16½ (X2), 17½ (X2), 18½ (X2) and 20½ (X1) days gestation. Each of these 12 cultures was examined daily by phase-contrast microscopy and was seen to be viable throughout the course of the experiment (Figs. 4.1 a-h). The cultures were maintained for approximately 10 days in hormone-free medium, followed by 5 days in medium containing 10 iu HCG/ml and a further 5 days in medium containing 0.5 mM cAMP.

Each day, the cultures medium was collected and replaced by 5 ml of fresh culture medium. The monolayers were not washed between daily medium changes as this was considered not to be necessary. No attempt was made to assay the concentration of HCG or cAMP at the end of each 24 hour culture period in these experiments. El-Khatim(1976) and Tashjian et al. (1973) have shown that HCG is not broken down in tissue culture.

1. Cell morphology in monolayer culture

After 1 day in monolayer culture (Fig.4.1 a-d) many of the cells had become attached to the floor of the tissue culture

Fig. 4.1. Phase-contrast photomicrographs of foetal rat testes in monolayer culture maintained at 37°C in 5ml of tissue culture medium in Falcon tissue culture flasks.

Fig. 4.1a&b. This culture was prepared from 24 foetal rat testes at 19½ days gestation and photographed after 1 day in tissue culture. Large angular cells have become attached and have spread out along the base of the culture flask. The small dark rounded cells have settled to the bottom of the culture medium but are not attached. Non-viable cells and less dense germ cells remain in suspension, and are seen as round white refractile blobs.

The two photographs indicate the varying plating density of cells within a single culture. (RFT 22/1/76).

(Phase-contrast X 100)

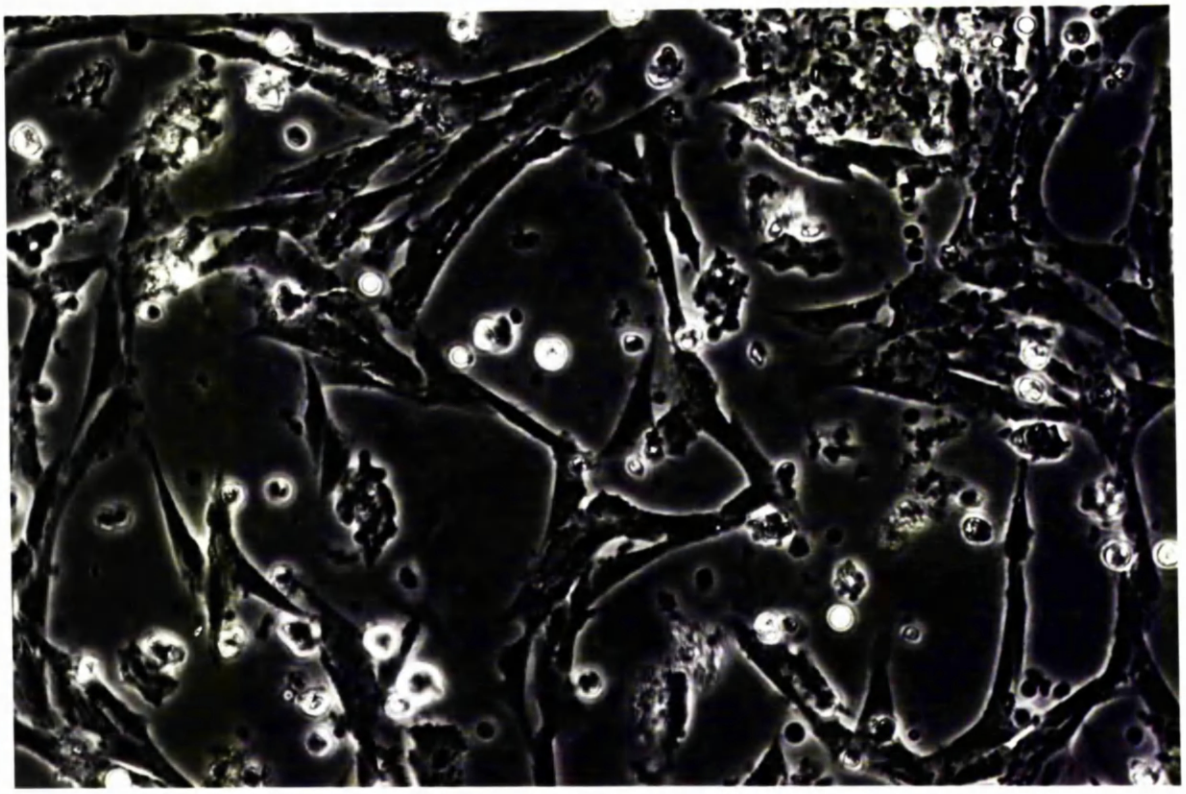


Fig. 4.1.a

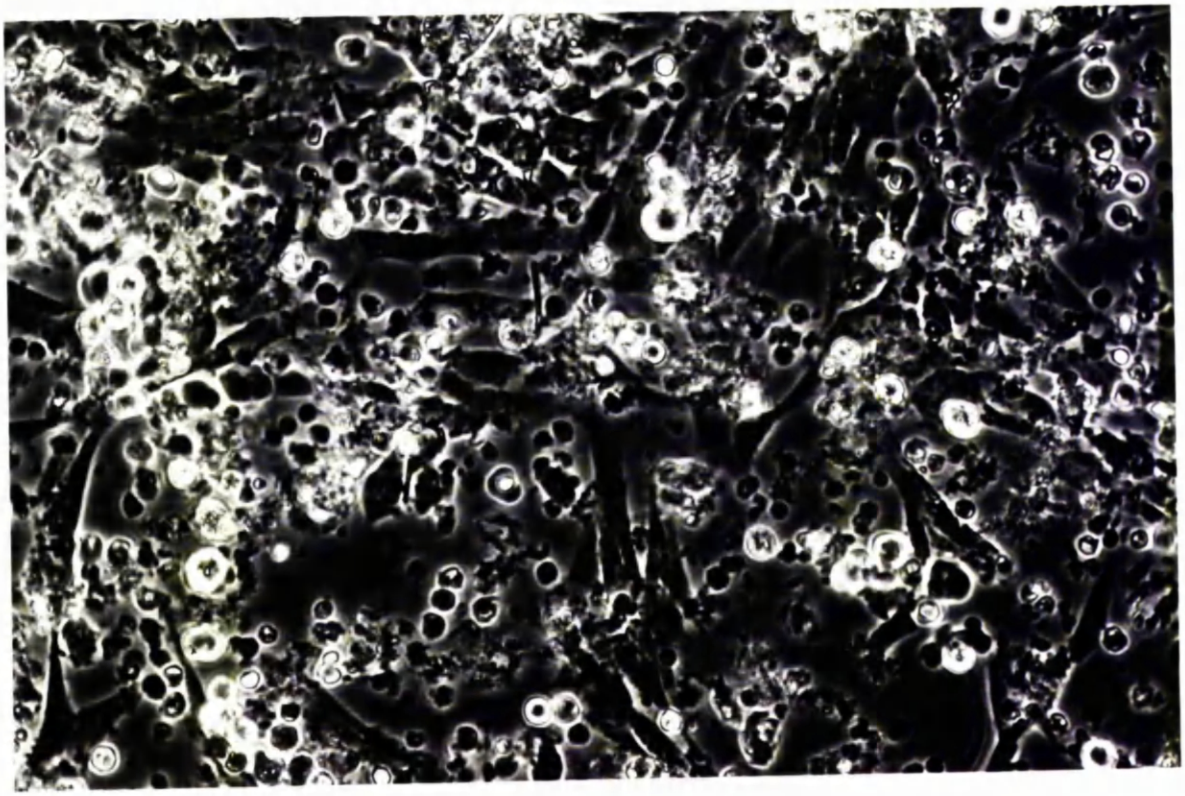


Fig. 4.1.b

Fig. 4.1. Phase-contrast photomicrographs of foetal rat testes in monolayer culture maintained at 37°C in 5ml of tissue culture medium in Falcon tissue culture flasks.

Fig. 4.1c. Culture prepared from 20 foetal rat testes at 18½ days gestation and photographed after 1 day in culture. Cells are spreading out along the floor of the flask from a multilayered refractile clump of cells forming an almost confluent monolayer. (RFT 21/1/76).

(Phase-contrast X 90)

Fig. 4.1d. Different region of the same culture photographed at the same time. This area is very sparsely populated with cells. (RFT 21/1/76).

(Phase-contrast X 85)

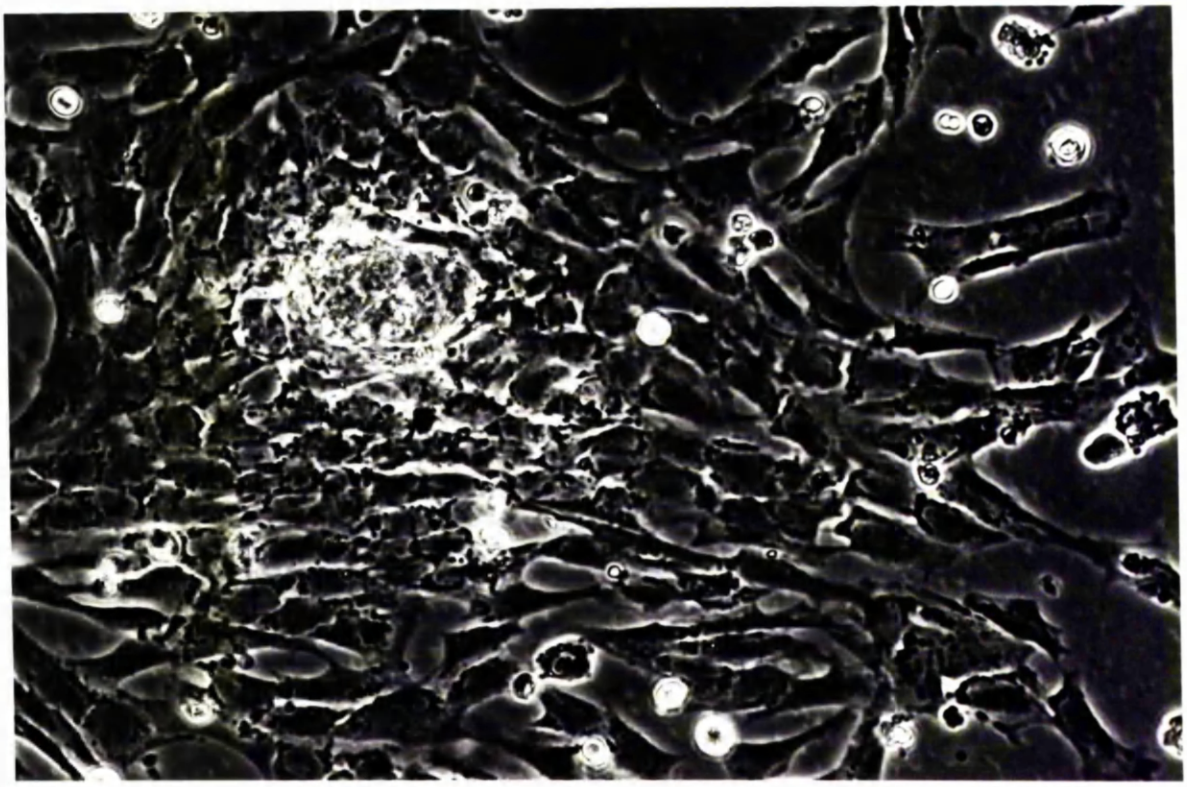


Fig. 4.1.c

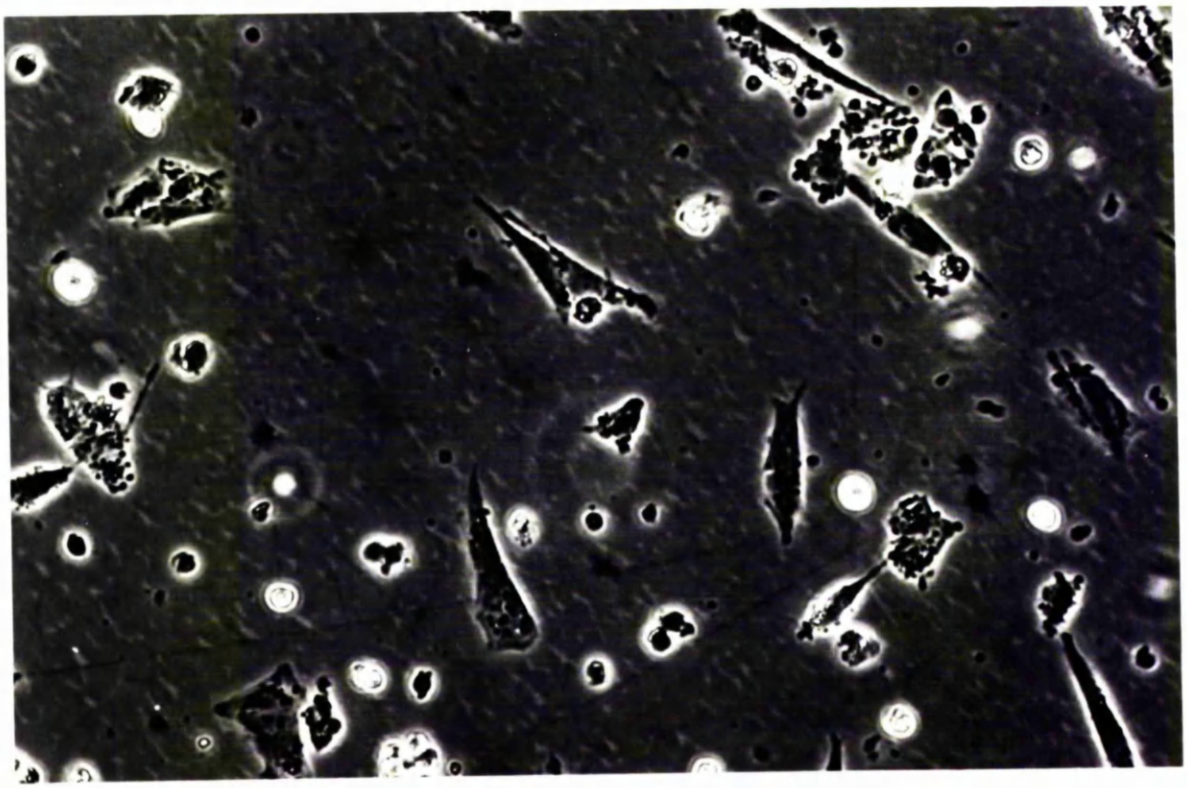


Fig. 4.1.d

Fig. 4.1. Phase-contrast photomicrographs of foetal rat testes in monolayer culture maintained at 37°C in 5ml of tissue culture medium in Falcon tissue culture flasks.

Fig. 4.1e. Culture prepared from 25 foetal rat testes at 19½ days gestation photographed after 5 days in culture. Most of the cells are adherent and have spread out to form a confluent monolayer. (RFT 24/6/77 : same as Fig. 2.2).

(Phase-contrast X 150)

Fig. 4.1f. Culture prepared from 28 foetal rat testes at 16½ days gestation photographed after 10 days in culture in medium with no hormone added, and a further 5 days in medium containing HCG. The cell morphology has not changed appreciably by the addition of HCG. (RFT 3/5/76A).

(Phase-contrast X 140)

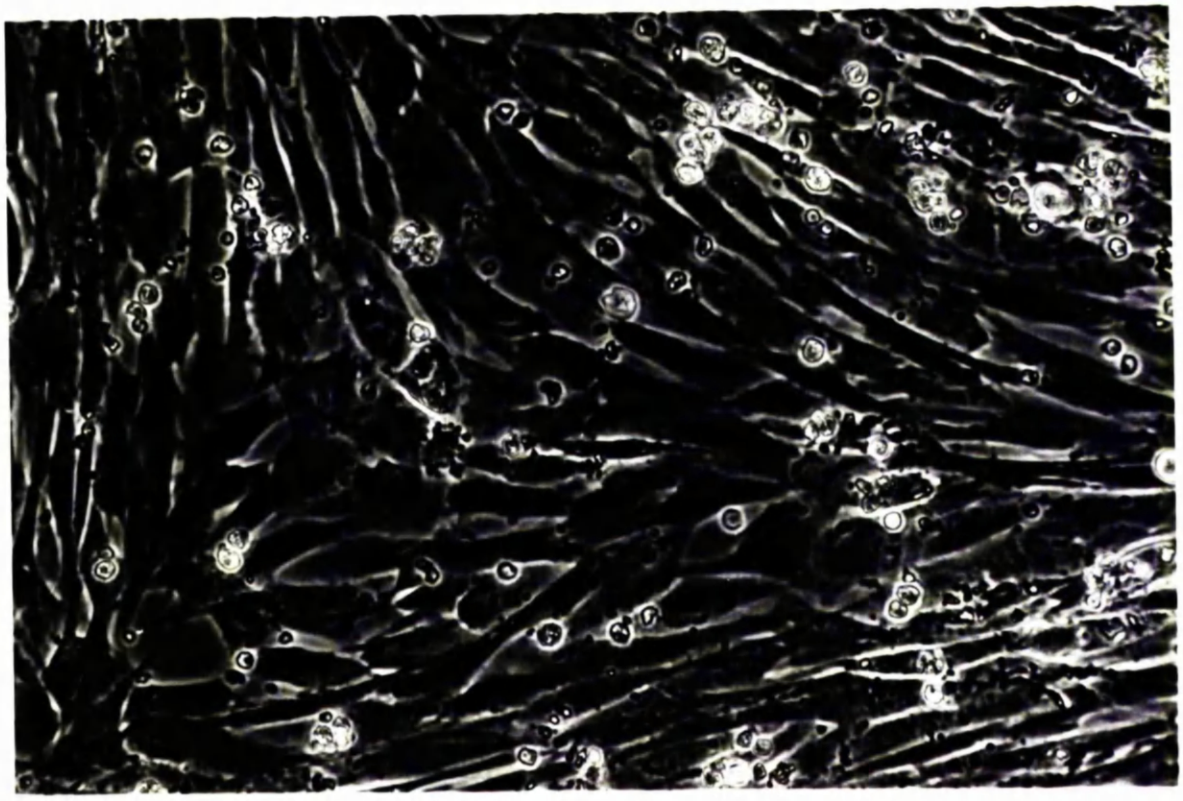


Fig. 4.1.e

Fig. 4.1.f

Fig. 4.1. Phase-contrast photomicrographs of foetal rat testes in monolayer culture maintained at 37°C in 5ml of tissue culture medium in Falcon tissue culture flasks.

Fig. 4.1g. Culture prepared from 28 foetal rat testes at 16½ days gestation (same culture as Fig. 4.1f) photographed after 6 days in medium containing cAMP following 10 days in non-hormonal medium and 5 days in HCG medium. Again, there is no appreciable change due to trophic stimulation. (RFT 3/5/76A). (Phase-contrast X 150)

Fig. 4.1h. Different region of the same culture photographed at the same time as Fig. 4.1g. Cell proliferation has been incompletely suppressed and in this field, the cells have heaped up into a refractile mass at the lower right hand corner. (RFT 3/5/76A).

(Phase-contrast X 140)

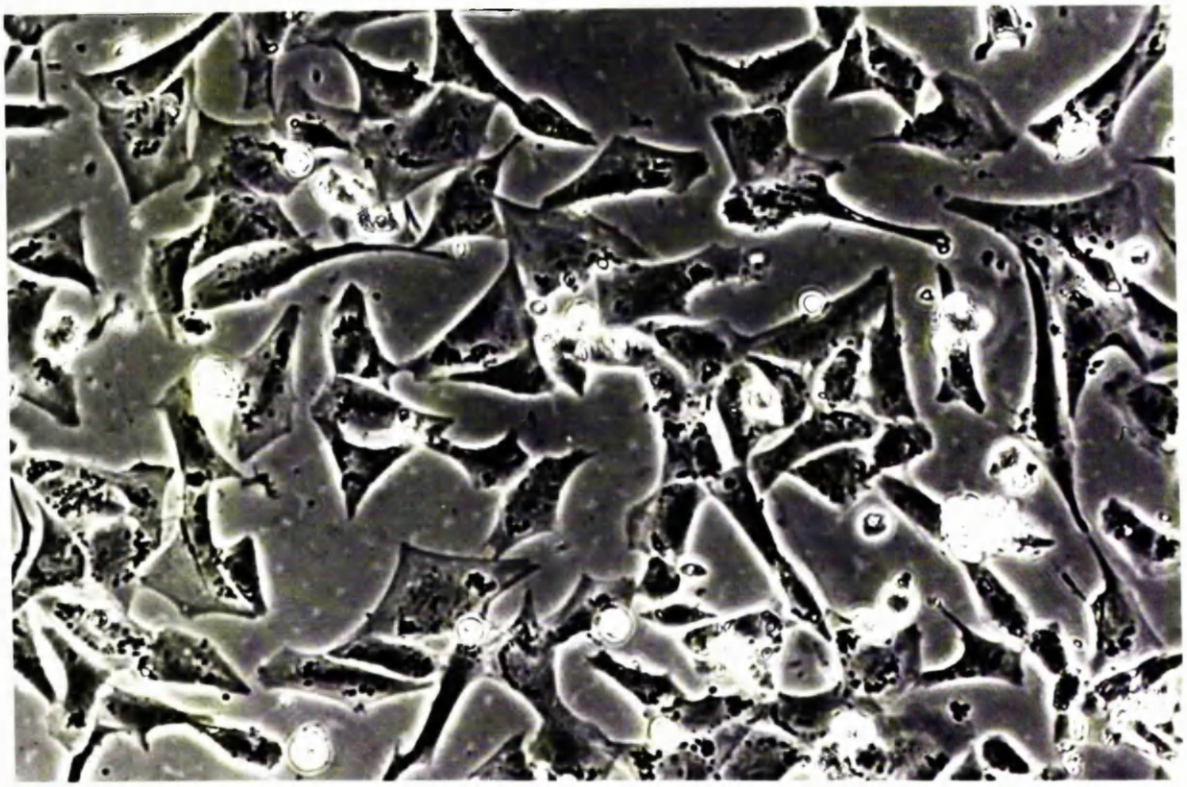


Fig. 4.1.g

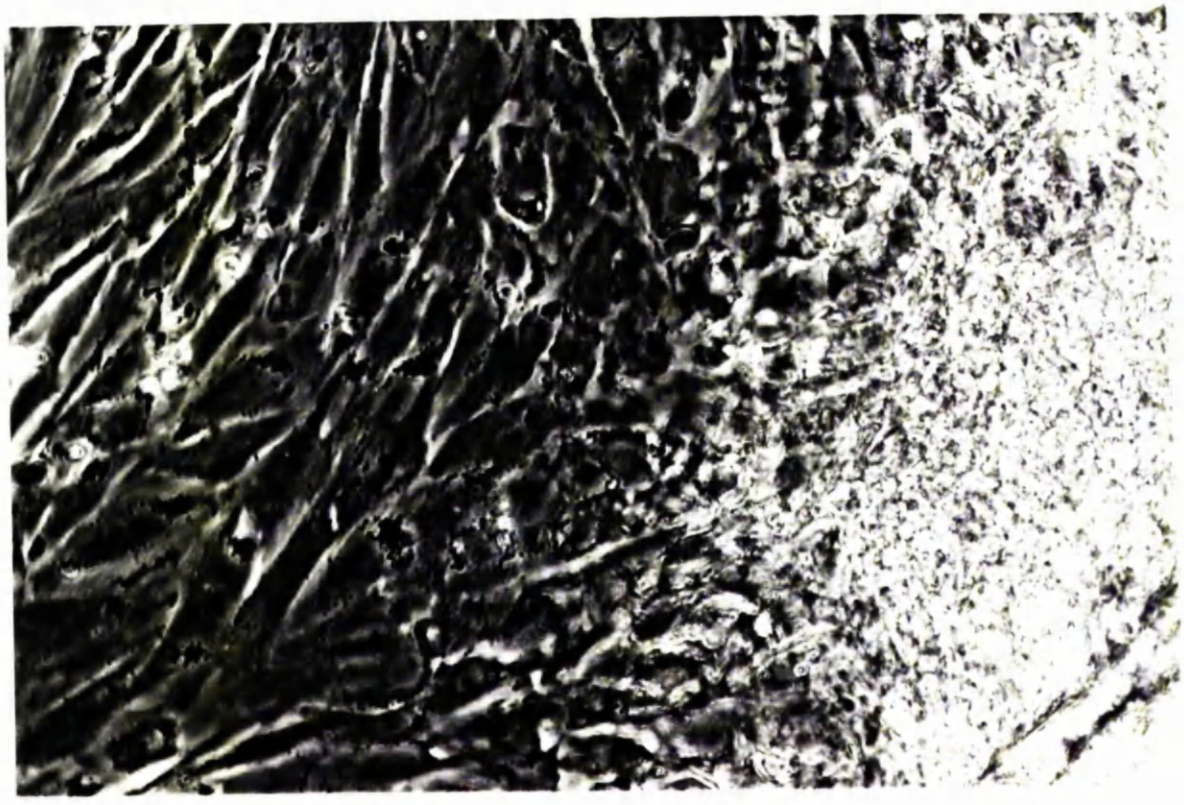


Fig. 4.1.h

flask and had spread out as flattened squames with many sharp cytoplasmic projections. Some cells remained in clumps (Fig. 4.1c), some were rounded in suspension (Fig. 4.1c), and others had become attached but remained rounded (Fig. 4.b). It is assumed that non-viable cells, and low density primitive germ cells remain in suspension and are removed when the culture medium is poured off: the interstitial cells and Sertoli cells are left attached to the flask.

The plating density of the cells in culture varied greatly from culture to culture, and in different parts of the same culture (Fig. 4.1 a-d). Different types of cells could not be identified by phase-contrast microscopy in particular interstitial cells and Sertoli cells could not be differentiated. Germ cells and fibroblasts were assumed to form only a small proportion of the total number of cells.

After several days in culture, most of the attached cells became flattened, and the cells in the clumps migrated out along the floor of the flask to become a monolayer (Fig. 4.1 e). At this stage, the morphology of the cultures remained relatively static and was not appreciably altered by the addition of either HCG (Fig. 4.1f) or cAMP (Fig. 4.1g) to the culture media. In some cultures however, suppression of cell division was not complete and proliferative activity resulted in piling up and stratification of cells, some of which became detached (Fig. 4.1h).

2. Endogenous steroid production

The daily endogenous production of progesterone and testosterone by foetal rat testes in monolayer culture was measured by radioimmunoassay (RIA) and is shown in Fig.4.2a-g.

Fig. 4.2. Daily endogenous secretion of progesterone and testosterone by monolayer cultures prepared from foetal rat testes of different gestational ages, and measured by radioimmunoassay.

HCG : Human chorionic gonadotrophin

cAMP : Monobutyryl cyclic adenosine monophosphate.

Fig. 4.2a. 14½ days gestation

Minimal amounts of progesterone and testosterone are produced by the unstimulated culture, or the culture after stimulation with HCG. Subsequent cAMP stimulation results in secretion of progesterone, but not testosterone.

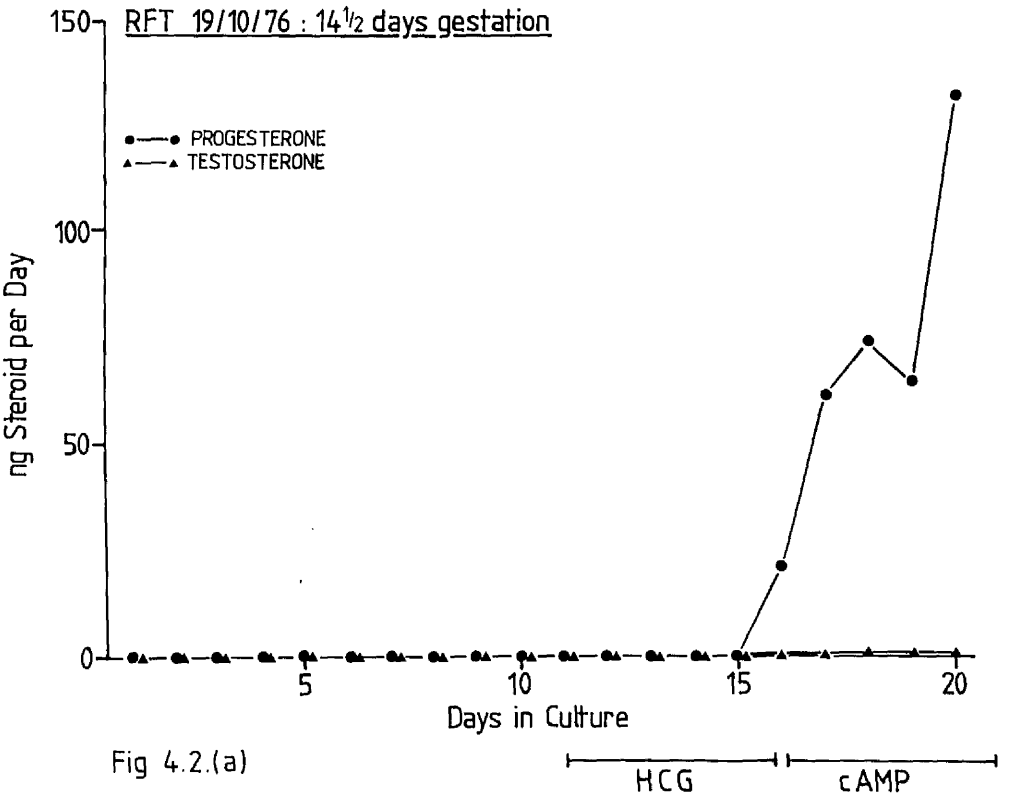

RFT 19/10/76 : 14½ days gestation
PROGESTERONE
TESTOSTERONE
ng Steroid per Day
150
100
50
0
5
10
15
20
Days in Culture
HCG
cAMP

Fig 4.2.(a)

Fig. 4.2. Daily endogenous secretion of progesterone and testosterone by monolayer cultures of foetal rat testes.

Fig. 4.2b_1 & 2b_2. 15½ days gestation

Testosterone is secreted in excess of progesterone in the first day of culture, but progesterone is the predominant steroid during the next few days. Thereafter, secretion of both falls to almost undetectable levels. Stimulation with cAMP, but not with HCG, causes increased progesterone output while testosterone secretion remains at baseline levels.

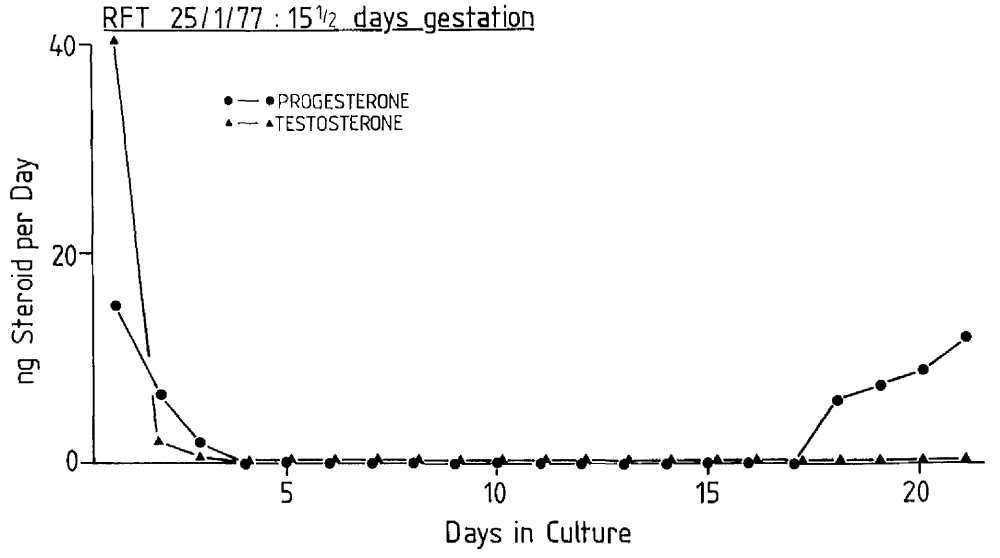


Fig 4.2. (b_1)

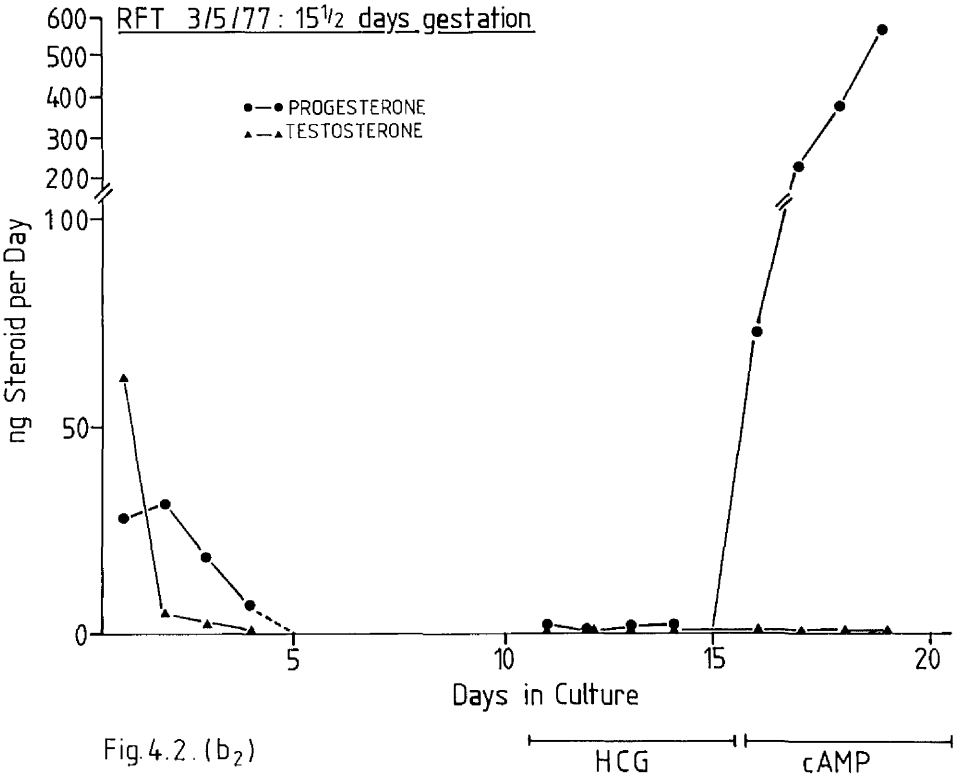


Fig. 4.2. (b_2)

Fig. 4.2. Daily endogenous secretion of progesterone and testosterone by monolayer cultures of foetal rat testes.

Fig. 4.2c_1 & 2c_2. 16½ days gestation

Testosterone is the main steroid secreted on day 1, but subsequently testosterone levels fall and progesterone output increases temporarily. By day 9, steroid output is at baseline levels and does not increase with HCG stimulation. Further stimulation with cAMP may cause resumption of progesterone secretion (2c_1) but this is not consistently seen.

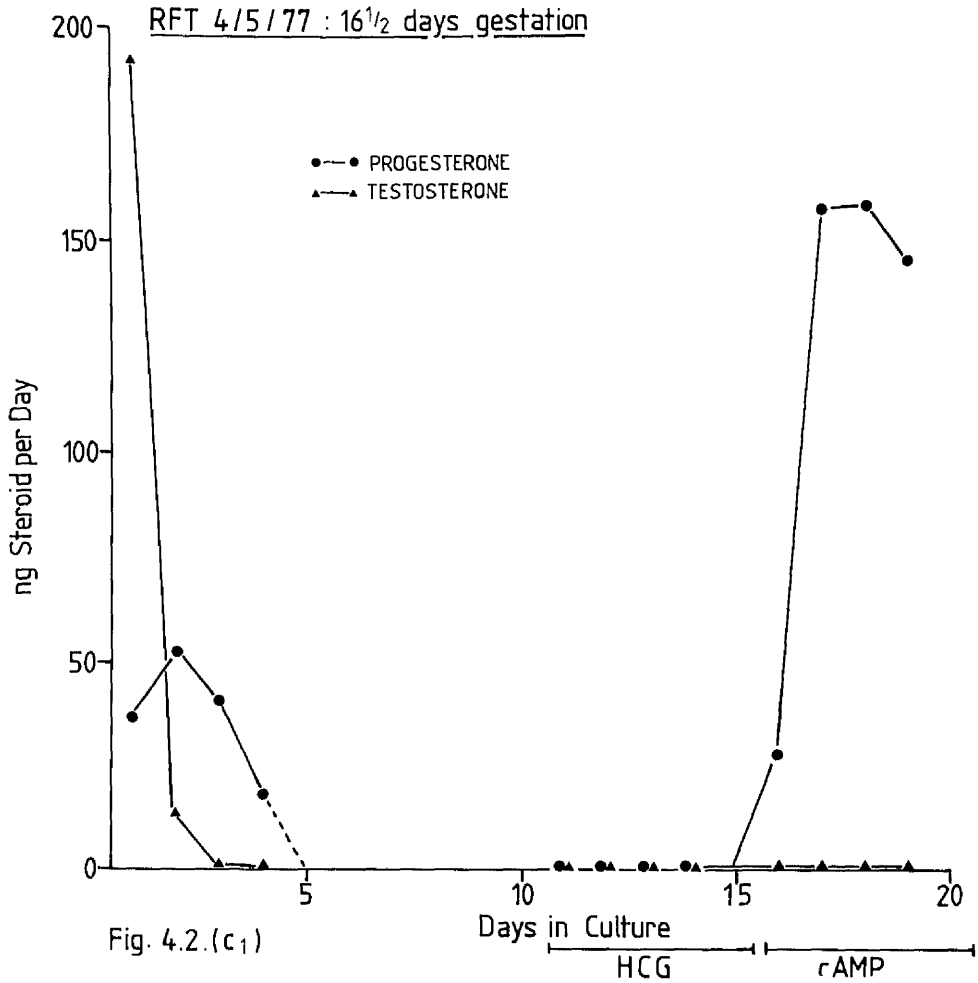


Fig. 4.2.(c_1)

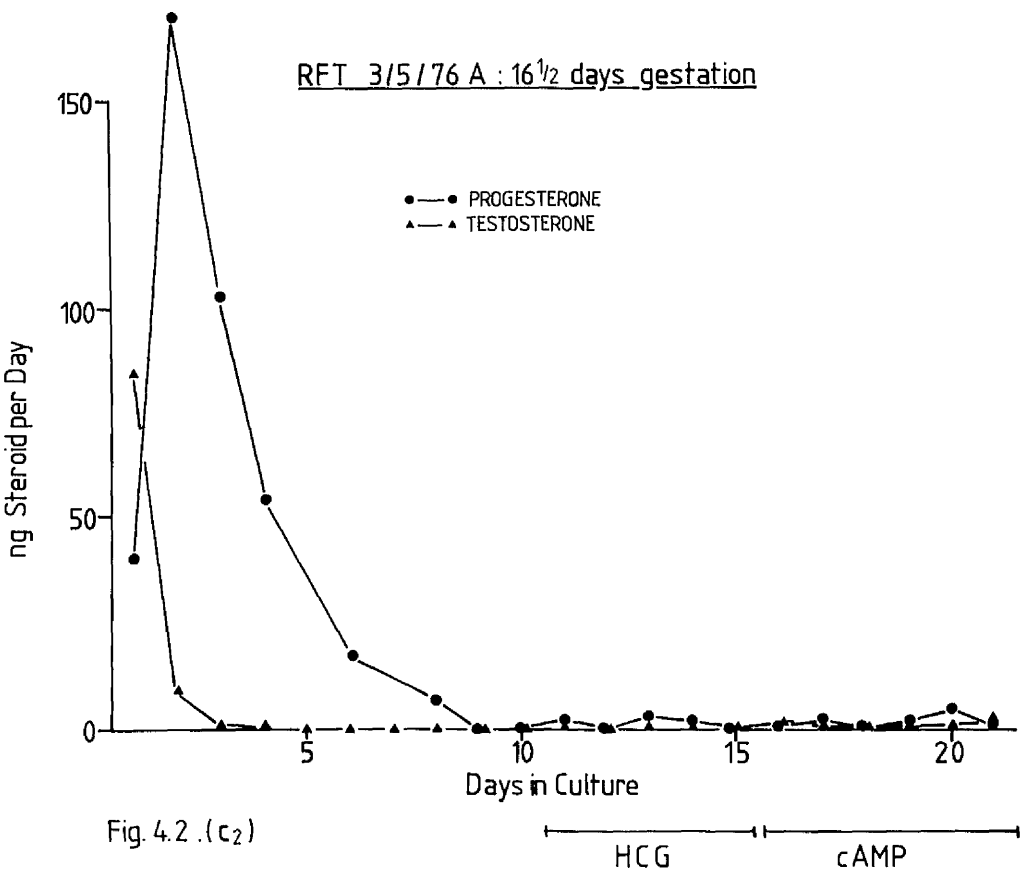


Fig. 4.2.(c_2)

Fig. 4.2. Daily endogenous secretion of progesterone and testosterone by monolayer cultures of foetal rat testes.

Fig. $4.2d_1$. 17½ days gestation

Production of testosterone, the major steroid at the start of the culture, falls rapidly and progesterone secretion increases temporarily. Then both fall to baseline levels. In this culture, there is no steroidogenic response to either HCG or cAMP.

Fig. $4.2d_2$. 17½ days gestation

This culture has a similar pattern of steroid production in medium without trophic stimulants. However, there is a small steroidogenic response to HCG stimulation, progesterone output being greater initially, but this is converted to testosterone in subsequent days. Stimulation with cAMP causes a much larger steroidogenic response, progesterone being the major secretory product.

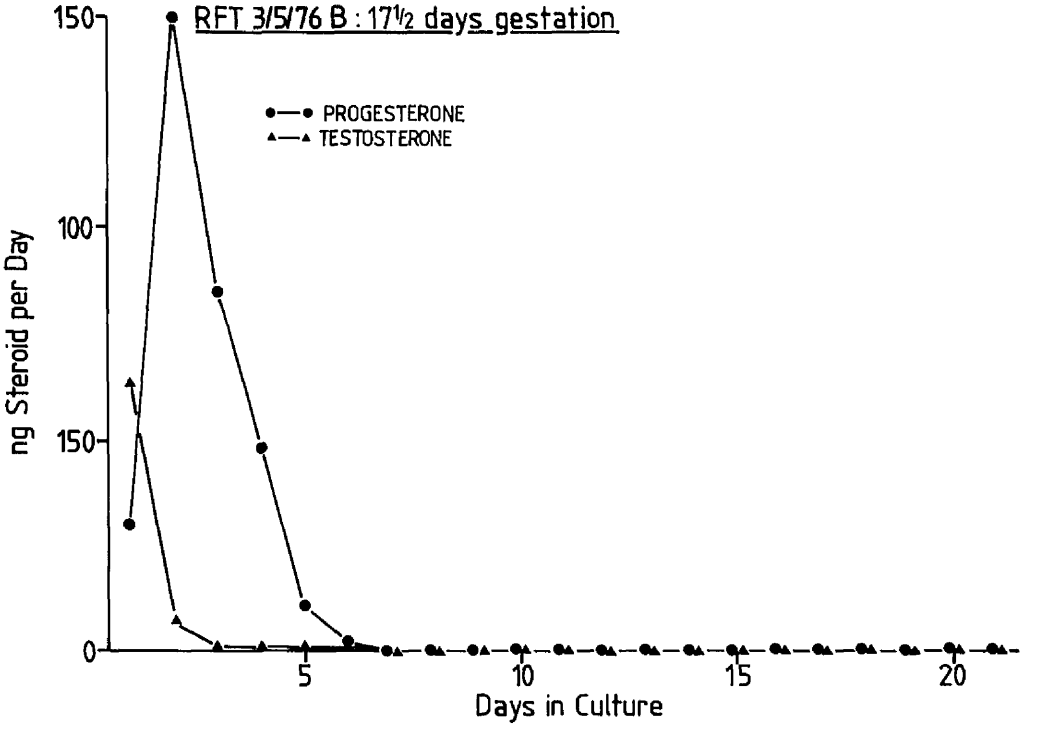


Fig. 4.2. (d_1)

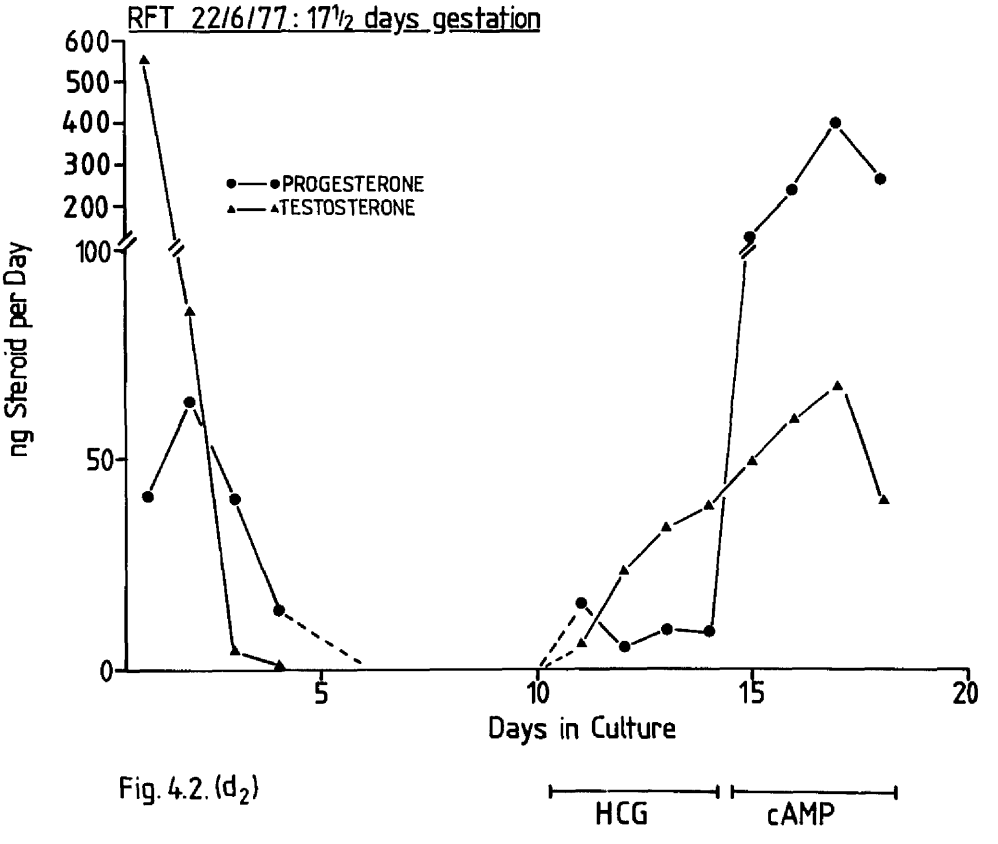


Fig. 4.2. (d_2)

Fig. 4.2. Daily endogenous secretion of progesterone and testosterone by monolayer cultures of foetal rat testes.

Fig. 4.2e_1 & 2e_2. 18½ days gestation

The initial pattern of steroid secretion is similar to that of the 15½, 16½ and 17½ days gestation cultures. Trophic stimulation has negligible effect in one of these cultures (2e_1). In the other (2e_2), HCG has no measurable stimulating action, but cAMP results in a marked increase in progesterone output.

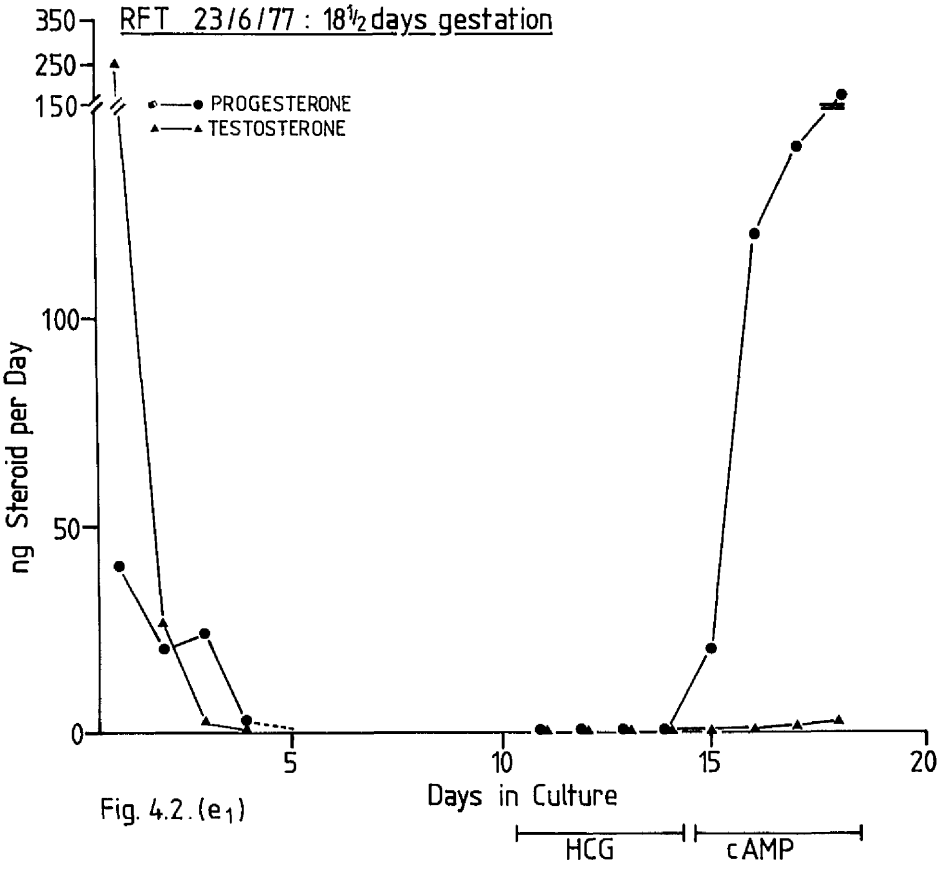


Fig. 4.2. (e_1)

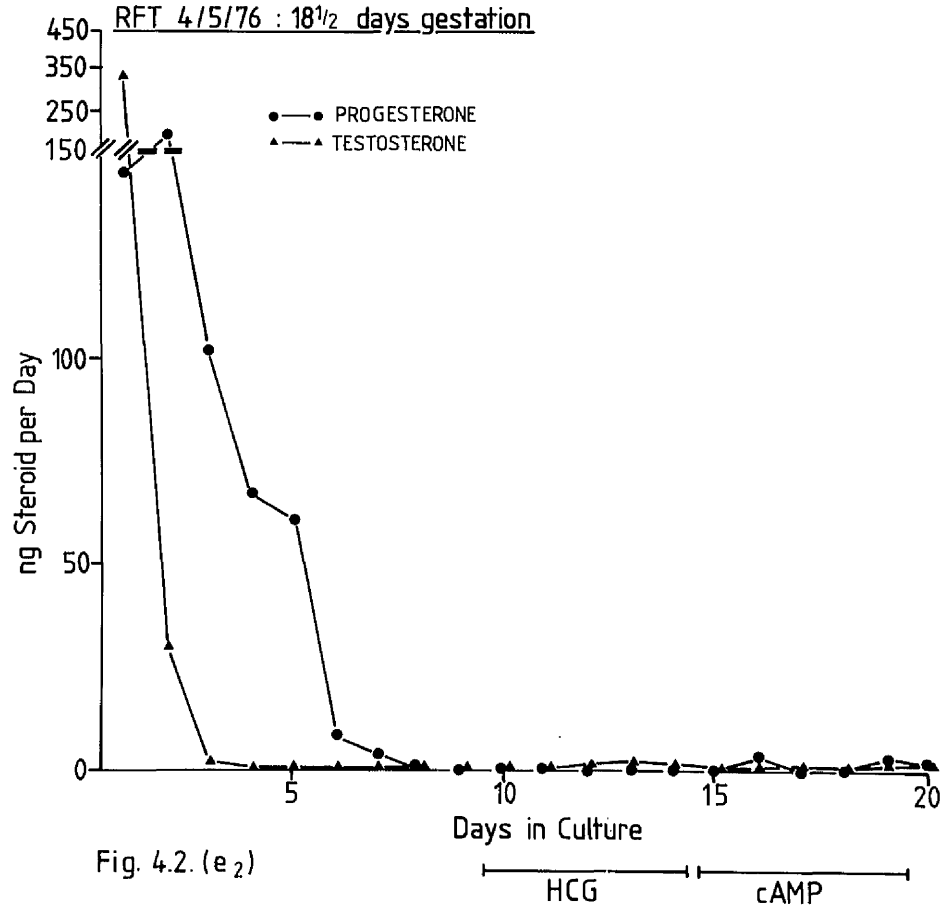


Fig. 4.2. (e_2)

Fig. 4.2. Daily endogenous secretion of progesterone and testosterone by monolayer cultures of foetal rat testes.

Fig. 4.2f_1 & 2f_2. 19½ days gestation

Testosterone is initially the major steroid hormone produced, but this rapidly falls to below the progesterone levels, then output of both diminishes to baseline. HCG stimulation causes an increase in progesterone secretion but this falls as testosterone production resumes and increases. Stimulation by cAMP results in increased output of both steroids, the progesterone response being more pronounced.

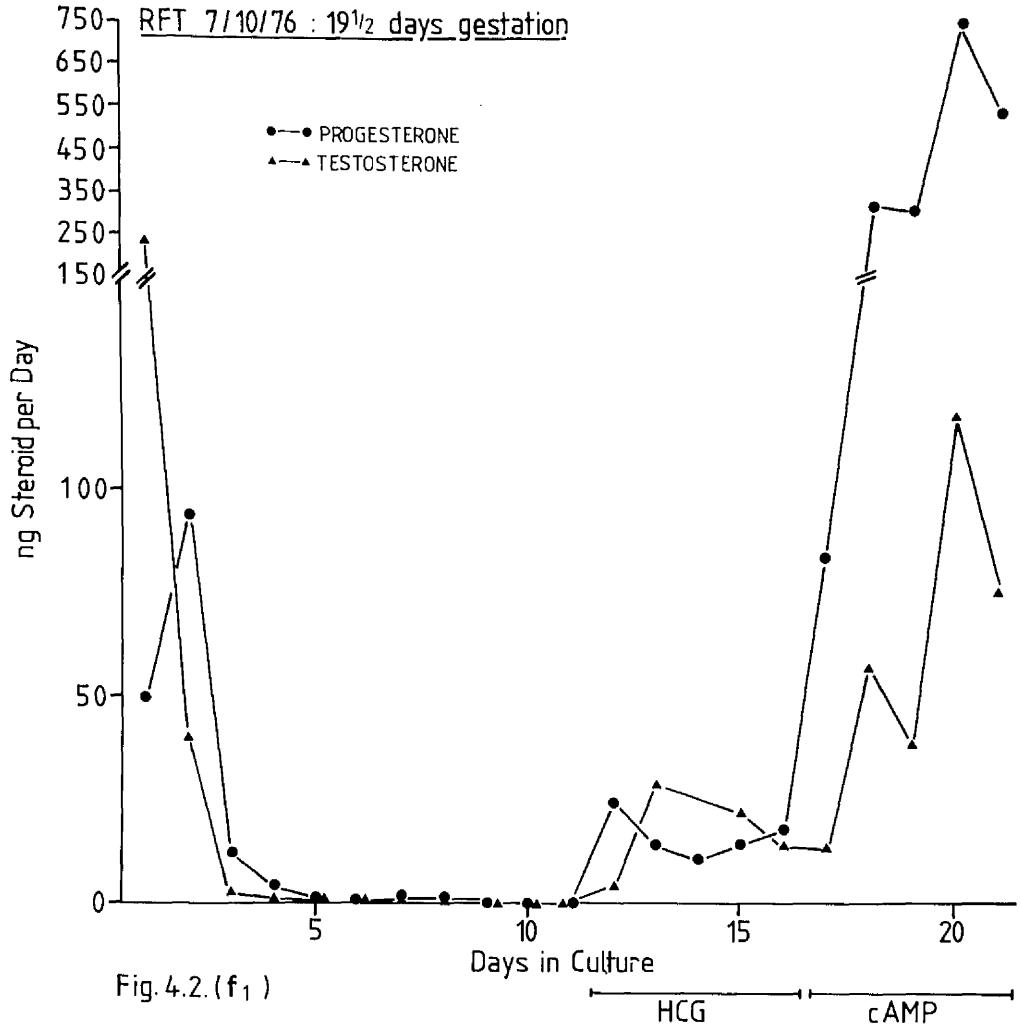


Fig. 4.2. (f_1)

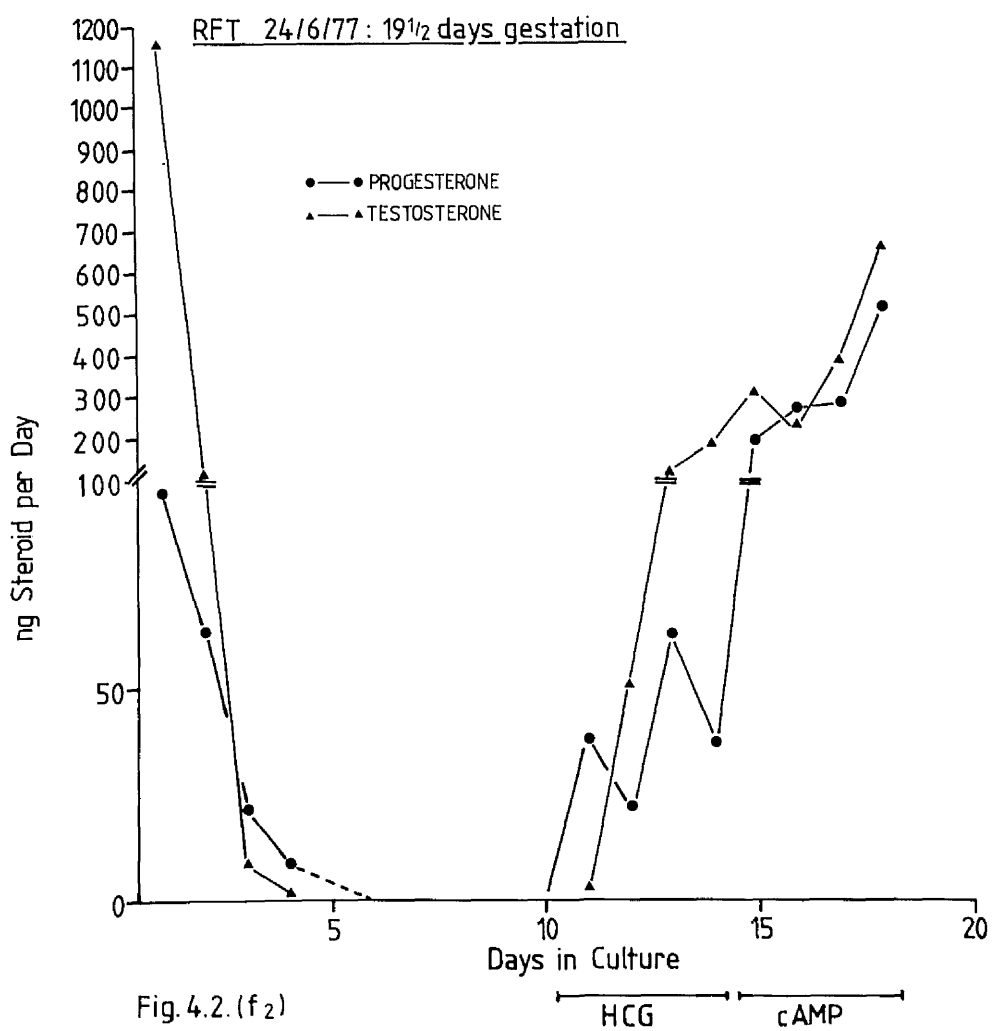


Fig. 4.2. (f_2)

Fig. 4.2. Daily endogenous secretion of progesterone and testosterone by monolayer cultures of foetal rat testes.

Fig. 4.2g. 20½ days gestation

Initial testosterone excess (lasting 1 day) is superceded by progesterone excess lasting for the next few days before both fall to baseline levels. HCG stimulation causes resumption of secretion of both steroids, progesterone reappearing first and remaining the predominant steroid. Cyclic AMP is a more potent stimulant causing increased output of both, the progesterone response being particularly marked.

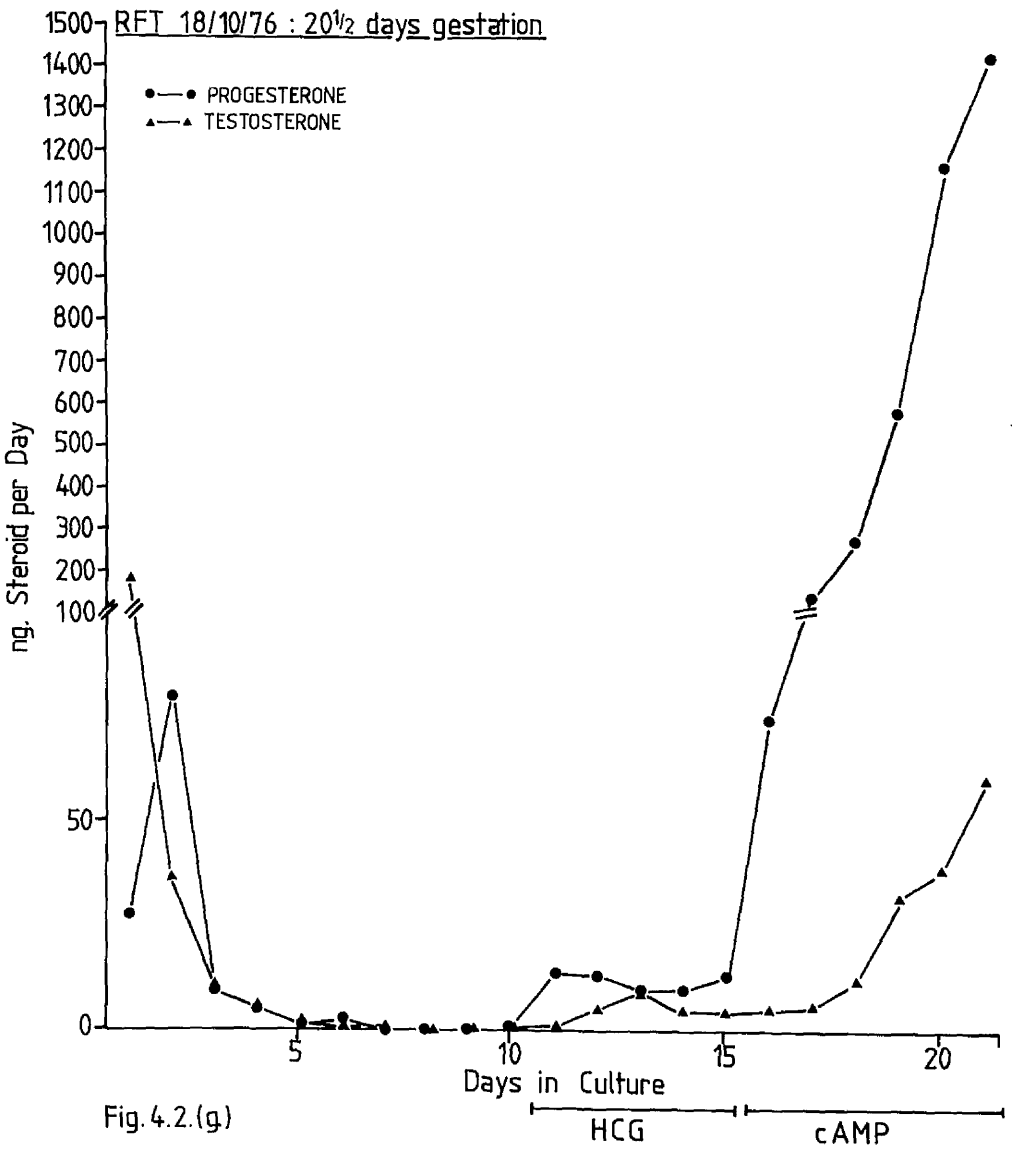

RFT 18/10/76 : 20½ days gestation
PROGESTERONE
TESTOSTERONE
ng. Steroid per Day
1500
1400
1300
1200
1100
1000
900
800
700
600
500
400
300
200
100
50
0
5
10
15
20
Days in Culture
HCG
cAMP

Fig. 4.2.(g)

In many instances, when RIA showed substantial steroid production, the identity of the steroids was checked by HPLC. The correlation between RIA and HPLC is shown in Table 4.I, and the steroids detected by HPLC are shown in Table 4.II.

In my hands, HPLC gave good separation of steroids and was good for identifying individual steroids. However, the precision of the method was not good and at best it could only be considered semi-quantitative. On the other hand, RIA was more sensitive and gave better quantitation but was less specific. Table 4.I shows that for most of the media there was good correlation between the results given by RIA and HPLC, but for other media marked discrepancies occurred. For example, in the monolayer culture prepared from 20½ day foetal rat testes, the culture media collected on days 2 and 16 contained appreciable amounts of progesterone as measured by RIA. However, progesterone was not detected in these media by HPLC. Table 4.II shows that these 2 culture media contained 20α-dihydroprogesterone which may conceivably have cross-reacted with the progesterone antibody. Such a cross-reaction,however, is unlikely since it has already been shown (Table 2.III) that 20α-dihydroprogesterone does not appreciably interfere in the progesterone RIA.

The graphs of steroid output (Fig. 4.2 a-g) show that in cultures prepared from testes of 15½ day and older foetal rats, testosterone is initially produced in greater amounts than progesterone: but, thereafter, testosterone output falls while progesterone output increases. After a few days, production of both steroids falls to very low baseline levels. Steroidogenesis can be reactivated by stimulation with cAMP and cultures prepared from older foetuses may also respond to HCG to a lesser degree.

Table 4.I. Daily endogenous production of testosterone and progesterone by foetal rat testes in monolayer culture as measured by radioimmunoassay * (RIA) and high pressure liquid chromatography (HPLC).

Gestational age of rat foetal testes from which monolayer cultures were prepared	Day of culture	Additive to culture medium	Endogenous steroid production (ng/ml) in vitro			
			Progesterone		Testosterone	
			RIA	HPLC	RIA	HPLC
$14\frac{1}{2}$ days	18	cAMP	74	19	0.5	10.5
	19	cAMP	65	108	ND	19
	20	cAMP	132	23	0.2	14
$18\frac{1}{2}$ days	1		145	ND	335	47
	2		190	ND	30	ND
	3		102	38	2.6	ND
	4		67	38	0.7	ND
	5		60	19	0.9	ND
$19\frac{1}{2}$ days	1		50	355	240	109
	2		94	43	40	16
	18	cAMP	312	186	57	17
	19	cAMP	300	23	38	8.4
	20	cAMP	740	450	117	132
	21	cAMP	533	345	75	78
$20\frac{1}{2}$ days	1		28	96	186	90
	2		80	ND	37	14
	16	cAMP	75	ND	5	ND
	17	cAMP	139	105	6	ND
	18	cAMP	272	184	12	42
	19	cAMP	586	414	32	63
	20	cAMP	1170	539	39	47
	21	cAMP	1420	1390	60	101

cAMP : monobutyryl cyclic adenosine monophosphate

ND : not detected

* In the RIA, dextran coated charcoal was used to separate the free and bound steroid

Table 4. II. Daily endogenous steroid production by foetal rat testes in monolayer culture as measured by high pressure liquid chromatography (HPLC).

Gestational age of rat foetal testes from which monolayer cultures were prepared	Day of culture	Additive to culture medium	Endogenous steroid production in vitro (ng/ml)				
			Prog	Testo	16αOHP	17αOHP	20αDHP
$14\frac{1}{2}$ days	18	cAMP	19	10.5	ND	ND	33
	19	cAMP	108	19	ND	42	64
	20	cAMP	23	14	33	ND	100
$18\frac{1}{2}$ days	1		ND	47	ND	ND	29
	2		ND	ND	89	ND	71
	3		38	ND	ND	ND	19
	4		38	ND	ND	ND	19
	5		19	ND	ND	ND	19
$19\frac{1}{2}$ days	1		355	109	19	121	ND
	2		43	16	ND	ND	109
	18	cAMP	186	17	23	80	168
	19	cAMP	23	8.4	ND	32	202
	20	cAMP	450	132	57	195	1020
	21	cAMP	345	78	28	131	694
$20\frac{1}{2}$ days	1		96	90	ND	61	46
	2		ND	14	ND	ND	207
	16	cAMP	ND	ND	ND	ND	74
	17	cAMP	105	ND	ND	ND	86
	18	cAMP	184	42	23	73	289
	19	cAMP	414	63	38	145	689
	20	cAMP	539	47	45	79	1210
	21	cAMP	1390	101	68	184	2000

cAMP : monobutyryl cyclic adenosine monophosphate

ND : not detected

16αOHP : 16α-hydroxyprogesterone

17αOHP : 17α-hydroxyprogesterone

20αDHP : 20α-dihydroprogesterone

* Full systematic nomenclature of steroids is given in Appendix I

When steroidogenesis is reactivated, progesterone production is detected several days before testosterone output is identified. In the culture prepared from 14½ day rat foetal testes (Fig. 4.2a) endogenous steroid output was not detected in the unstimulated, or HCG-stimulated, cultures. However, stimulation with cAMP resulted in progesterone biosynthesis.

The results from the HPLC (Table 4.II) show that cultures also produced 20α-dihydroprogesterone, and a marked increase in production of this steroid was noticed after cAMP stimulation. Small amounts of 16α-hydroxyprogesterone and 17α-hydroxyprogesterone were also detected, but trophic hormonal stimulation of the cultures showed a less marked effect on the production rates of these steroids.

3. Monolayer cultures from testicular feminisation

Monolayer cultures of interstitial cells from testes from patients with testicular feminisation were prepared and maintained. The culture media were assayed for testosterone and progesterone. The daily endogenous production and output of these steroids are shown in Figs. 4.3 & 4.4 which also show the effect of stimulation with HCG and cAMP.

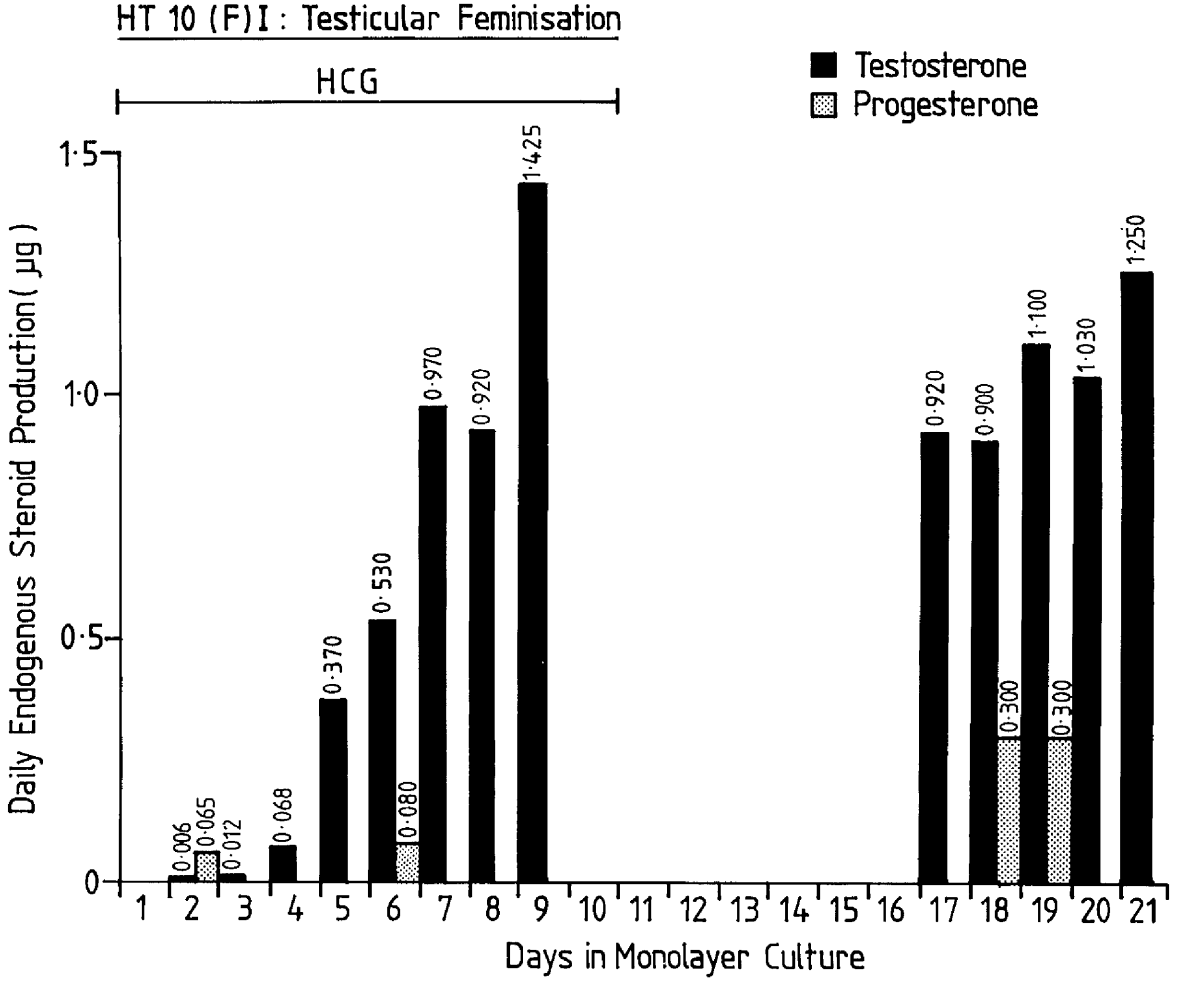
Cultures prepared from one patient (Fig. 4.3) initially produced very low levels of testosterone, but synthesis of this steroid increased over the next 9 days before falling off slightly. Progesterone was initially produced in greater quantities, and although output of this steroid also increased with the age of the culture, by the 6th day testosterone output exceeded that of progesterone and thereafter remained the major product detected.

Fig. 4.3. Daily endogenous secretion of progesterone and testosterone from monolayer cultures prepared from human testicular feminisation testes.

Seminiferous tubules were drawn out leaving interstitial cells which were used to prepare two identical monolayer cultures, each in Falcon tissue culture flasks with 5ml of tissue culture medium. The medium was changed daily and endogenous progesterone and testosterone production determined by radioimmunoassay.

Fig. 4.3a. Monolayer culture maintained for 10 days in HCG-medium, then 10 days in hormone-free medium. [HT 10(F) I].

Fig. 4.3b. Monolayer culture maintained for 10 days in hormone-free medium, then 10 days in HCG-medium. [HT 10(F) II].


HT 10 (F)I : Testicular Feminisation
HCG
Testosterone
Progesterone
Daily Endogenous Steroid Production(µg)
1·5
1·0
0·5
0
0·006
0·065
0·012
0·068
0·370
0·530
0·080
0·970
0·920
1·425
0·920
0·900
0·300
1·100
0·300
1·030
1·250
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
Days in Monolayer Culture

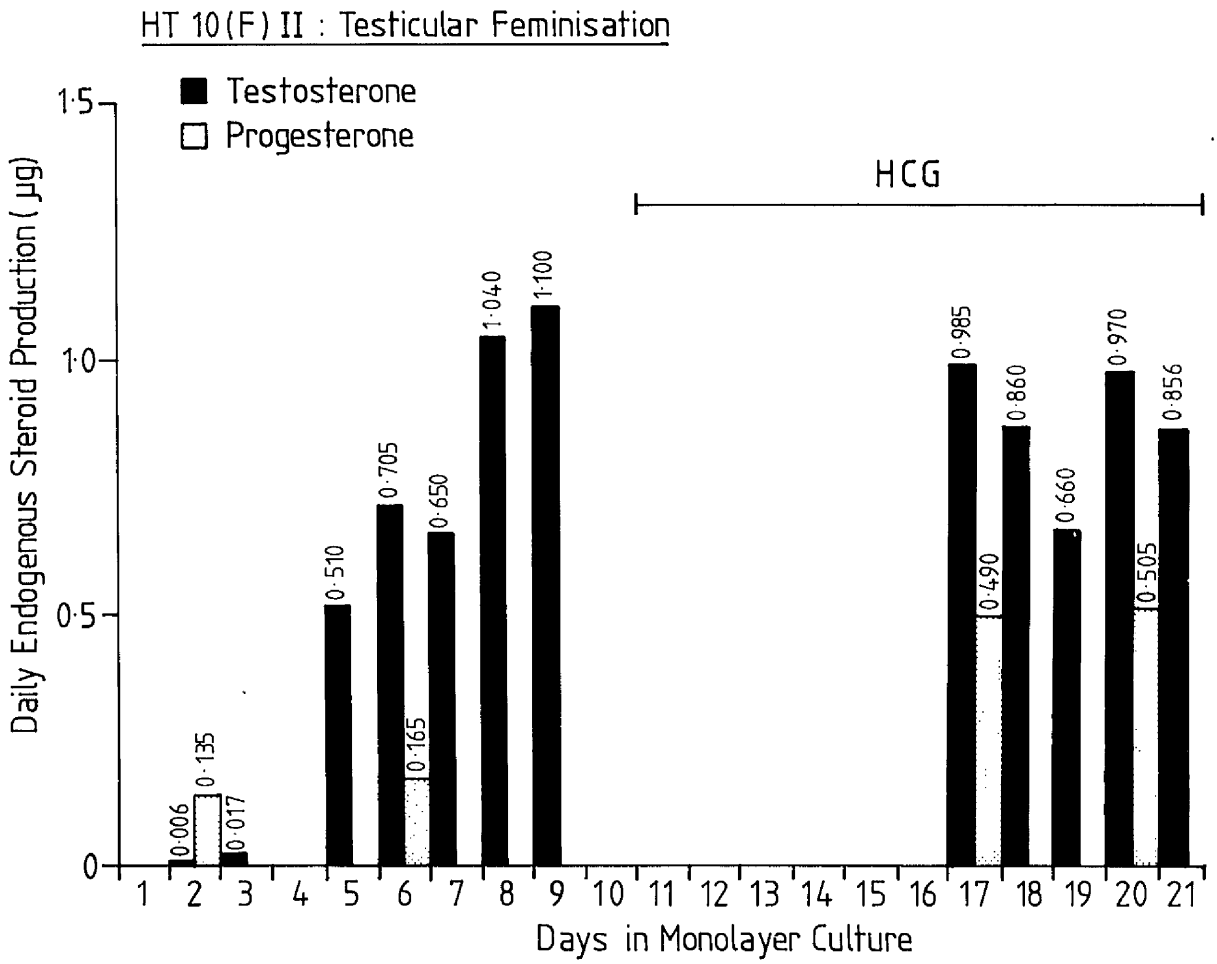

HT 10(F) II : Testicular Feminisation
Testosterone
Progesterone
HCG
Daily Endogenous Steroid Production (µg)
1·5
1·0
0·5
0
0·006
0·135
0·017
0·510
0·705
0·165
0·650
1·040
1·100
0·985
0·490
0·860
0·660
0·970
0·505
0·856
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
Days in Monolayer Culture

Fig. 4.4. Daily endogenous secretion of progesterone and testosterone from a monolayer culture prepared from human testicular feminisation testes.

A monolayer culture of interstitial cells was prepared as in Fig. 4.3. The culture was maintained for 10 days in hormone-free medium, then 5 days in HCG-medium followed by a further 6 days in cAMP-medium. [HT 26(M)].

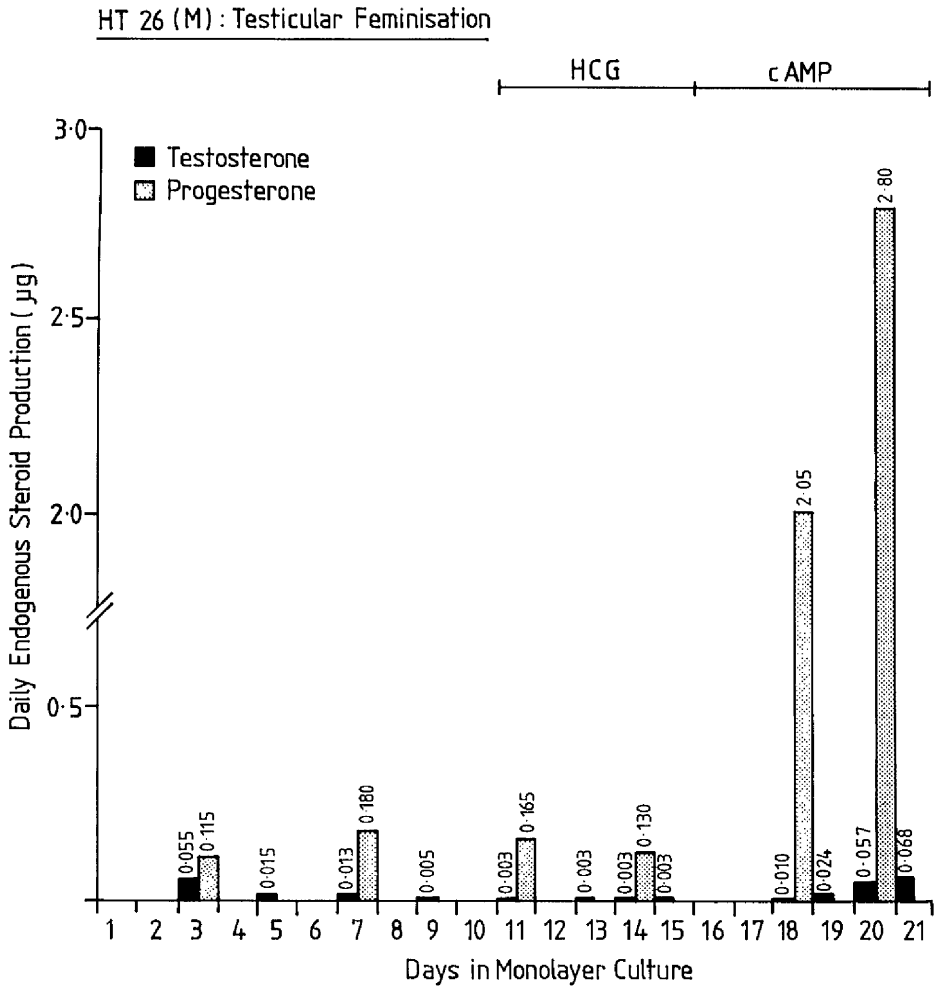

HT 26 (M) : Testicular Feminisation
HCG
cAMP
Testosterone
Progesterone
Daily Endogenous Steroid Production (µg)
3·0
2·5
2·0
0·5
0·055
0·115
0·015
0·013
0·180
0·005
0·003
0·165
0·003
0·003
0·130
0·003
0·010
2·05
0·024
0·057
2·80
0·068
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
Days in Monolayer Culture

Fig. 4.4.

HCG had little effect on steroidogenesis.

Monolayer culture of the other patient produced progesterone in greater quantities than testosterone at all stages measured (Fig. 4.4). Testosterone production, in this case, started high and fell to very low levels whereas progesterone secretion continued at a much higher rate. HCG had negligable stimulatory effect. On the other hand, cAMP caused significant increase in testosterone output, and increased the secretion rate of progesterone by a factor of 10 to 20.

B. DISCUSSION

In discussing the physiological significance of the above results, it must be assumed that the *in vitro* function of the cells resembles the *in vivo* situation, but this can only be valid if the effects of tissue culture variables on cell function are known, and the degree of maturation of cells *in vitro* can be assessed.

1. Tissue culture variables

Neville & O'Hare (O'Hare *et al.*, 1978) have examined the effects of cell density, and the presence or absence of trophic hormone (ACTH), on the steroidogenic function of adreno-cortical cells in monolayer culture. The pattern of endogenous steroid production is affected by both these parameters. In the present series, difficulties precluded an accurate control of cell density of monolayer foetal testicular cells; and the proportion of steroid-producing cells (probably mainly interstitial cells of Leydig) to other types of cells was not known and could not be assessed or controlled.

The cell density of culture is affected by the rate of cell death and cell proliferation, and the steroidogenic function of cells may differ in various parts of the cell cycle. For this reason, an attempt was made to produce non-proliferative cell cultures by restricting the glutamine available to the cells. Unfortunately, too meticulous restriction of glutamine proved damaging to the cells, and a small amount had to be added to maintain cell viability. This resulted in some proliferative activity, more marked in some cultures than in others.

The lack of proper control of tissue culture variables may explain why cultures prepared from foetal testes of the same gestational age do not always respond identically.

2. Cell maturation in vitro

The organ culture experiments (vide supra) clearly demonstrate that foetal gonads and foetal genital tracts continue to mature and differentiate in vitro. It is not possible in the present monolayer culture experiments, to state that cells explanted from foetal testes at a given gestational age did - or did not - maintain the function of cells at that gestational age throughout the course of the culture. Therefore, the steroidogenic function of cells after several days in culture may - or may not - represent the function of cells at the gestational age of explantation.

3. Similarities between in vitro and in vivo function

There is abundant evidence to show that foetal testes produce androgenic steroids which masculinise the male genitalia in utero. Freshly explanted monolayer cultures of foetal rat testes from 15½ or more days gestation foetuses also produce androgens (testosterone), but androgenic production ceases in a non-hormonal environment (Fig. 4.2). Steroidogenesis can be re-stimulated by trophic hormones (HCG) or cyclic AMP. This is similar to the in vivo situation where trophic hormones from the foetal pituitary and placenta act on the foetal testes. Therefore, at least in certain respects, the cells in vitro mimic the function of cells in vivo.

O'Hare and his co-workers (see review by O'Hare et al., 1978) demonstrated that adrenocortical cells in monolayer culture are very similar in function to their activity in vivo, especially with respect to their response to trophic hormone. However, testicular interstitial cells in monolayer culture lose many of their in vivo characteristics. Cultures prepared from adult rat testes, and adult prepubertal and foetal human testes lose their responsiveness to HCG and LH (Khatim & O'Hare 1976; O'Hare et al., 1976). Adult rat and human testicular cells in monolayer culture lose their ability to secrete testosterone because of a decline in 17α-hydroxylase and C $^{17-20}$ desmolase activities (Khatim & O'Hare, 1976; O'Hare et al., 1976); stimulation by cAMP subsequently increases steroid output but does not reactivate these enzymes. Therefore, large quantities of progesterone and its metabolites are formed.

Cultures prepared from testicular feminisation testes continue to produce testosterone, but these cultures also lose their responsiveness to HCG (Figs.4.3 & 4.4). Stimulation with cAMP increases testosterone output, but progesterone secretion is stimulated to a much greater extent.

The experiments described in this thesis demonstrate that monolayer cultures prepared from foetal rat testes in some respects behave like cultures prepared from other types of testes, but in other respects, the rat testes in culture have certain properties peculiar to themselves. They are the only cultures to retain some responsiveness to HCG. Also, some of the cultures of foetal rat testes retain the ability to secrete testosterone in quantities in the same order of magnitude as progesterone (Fig.4.2 d_2, f_1, f_2,) when stimulated by cAMP, and this property has not been observed in cultures of other types of testes.

It can therefore be claimed that foetal rat testes in monolayer culture retain their *in vivo* properties and function better than other types of testes in culture.

4. Physiological significance of steroidogenesis by monolayer cultures

The biosynthesis of testosterone by the foetal testes can be considered as occurring in 2 stages: firstly, conversion of acetate to cholesterol and subsequent cholesterol metabolism by the enzymes C20:22 desmolase and 3β-HSD-I to produce pregnenolone and progesterone; and secondly, conversion of progesterone by the enzymes 17α-hydroxylase, 17 desmolase, and 17β-hydroxysteroid dehydrogenase forming respectively 17α-hydroxy-progesterone, androstenedione and testosterone (Fig. 1.3). This second stage may utilise the Δ^5- pathway (pregnenolone, 17α-hydroxypregnenolone, dehydroepiandrosterone) thus by-passing progesterone, and requiring a 3β-HSD-I isoenzyme system acting preferentially on DHA (dehydroepiandrosterone) rather than pregnenolone.

If the full complement of enzymes is present, testosterone is produced; but if only the first stage enzymes are functioning, progesterone is produced. It would appear that foetal rat testicular cells in monolayer culture rapidly lose the ability to convert progesterone to testosterone. Therefore, in the first few days in culture there is a diminution of testosterone output and an increase in progesterone output. This is similar to the decline in activity of 17α-hydroxylase and C^{17-20} desmolase described by Khatim & O'Hare (1976) in monolayer cultures of adult rat testicular interstitial cells.

The present experiments show that shortly after the foetal testicular cells lose their ability to convert progesterone to testosterone, the enzymes required for progesterone biosynthesis also become deactivated and steroid secretion falls below detectable levels.

Restimulation of steroidogenesis can be produced by the addition of HCG or cAMP to the culture medium, and in such instances, reactivation of progesterone biosynthesising enzymes occurs before the enzymes required for testosterone are active. Therefore, increase in progesterone output is detected initially, followed by an increase in testosterone production. This sequence of events may also occur *in vivo*. If so, when the foetal testis first secretes steroid hormones, progesterone will be released, and later, progesterone will be converted to testosterone before being released. The histochemical studies of Baillie and his co-workers (*vide supra*) support this sequence of events, and suggest that progesterone is synthesised at the time of Mullerian duct inhibition, and testosterone is released at the time of Wolffian duct stabilisation.

Steroidogenesis by steroid-producing cells in monolayer culture has been examined extensively over the last few years by members of our department (O'Hare *et al.*,1978) using adrenocortical cells, and more recently testicular cells. Monolayer cultures are used in preference to organ cultures because the exact cellular environment can be more readily controlled, therefore the exact requirements for normal cellular function can be assessed. However, the stringent requirements needed for normal function makes it more difficult to reproduce the *in vivo* situation in monolayer culture.

The steroidogenic activity of monolayer cultures prepared from human adult, prepubertal, neonatal or foetal testes, and from rat adult and prepubertal testes have been examined (O'Hare et al., 1978). Monolayers of rat foetal testes have not been previously described. There are many problems involved in preparing monolayer cultures from foetal rat testes, mainly because of (a) the difficulty in producing rat foetuses of an exact gestational age on a certain date; and because of (b) the small size of individual rat foetal testes necessitating a large number of testes being used in the preparation of one culture. Quantitation of tissue and control of cell density in culture is therefore very difficult, and it is not possible to determine the proportion of steroid-producing cells to non-steroidogenic cells in individual cultures. However, monolayer cultures were prepared and the cells remained viable for the duration of the culture periods used (3 weeks, or longer), and during this time endogenous steroidogenic activity was demonstrated.

The experiments described in this thesis demonstrate that foetal testicular cells from rats differ from human foetal testes with respect to steroidogenesis in monolayer culture. Rat foetal testicular cells rapidly cease producing testosterone, and shortly after, synthesis of progesterone also diminishes. In some cases, steroidogenesis recommences after HCG stimulation in vitro, but this effect is inconsistent. Stimulation with cAMP produces a much greater and more consistent steroidogenic response.

Cyclic AMP acts as a "second messenger" for trophic hormones. The trophic hormone binds to specific receptors on the target cell membrane and this stimulates cAMP production

which in turn activates intracellular activity. Testicular cells in monolayer culture lose their responsiveness to HCG, but cAMP is still active in stimulating cell metabolism and steroid output. This indicates that although testicular cells in monolayer culture are still capable of synthesising steroids if the intracellular environment is suitable, the cells lose their capacity to respond to a specific trophic hormone. This result is rather surprising because adrenocortical cells in monolayer culture retain their responsiveness to ACTH, a trophic hormone which also acts through cAMP as the second messenger. Rat foetal testes are unlike other types of testes examined because in some cultures they do show a small response to HCG.

It is clear, therefore, that the rat foetal testicular cells in monolayer culture in the present series of experiments retain steroidogenic activity *in vitro*, but there are differences between the *in vitro* and *in vivo* pattern of steroid hormone output. For this reason, *in vivo* activity cannot be elucidated with certainty from these results. However, some changes in the *in vitro* activity may reflect particular aspects of *in vivo* properties. For example, in the culture prepared from the 14½ day foetal rat testes (at a time when MIF is produced but androgens are not required for Wolffian duct stabilisation: see Fig.1.2) stimulation of the monolayer culture resulted in synthesis of progesterone but conversion of progesterone to testosterone was not evident (Fig 4.2a). This may imply that at 14½ days gestation, the foetal rat testis is capable of producing progesterone but not testosterone.

These results support the hypothesis that progesterone may be involved in Mullerian duct inhibition, and as the testis matures in the foetus, output of this Mullerian inhibitory factor

falls because of conversion to androgenic steroids which are required at a later date for Wolffian duct stabilisation.

The results of Picon (1976b) indicate that cAMP does not have Mullerian duct inhibitory activity. On the contrary, it prevents the Mullerian inhibitory action of the foetal testis in organ culture. Picon concludes that cAMP probably has a direct stabilising effect on the Mullerian duct causing its maturation, and therefore interferes with testicular MIF at the target organ level. However, it is also possible that cAMP stimulates the testicular cells to metabolise MIF into a product devoid of Mullerian inhibitory activity. Perhaps the cAMP stimulates conversion of progesterone into testosterone.

If progesterone is required for Mullerian duct inhibition, then this postulate must not conflict with known facts concerning sexual development in various syndromes which result in intersexuality.

In the syndrome of testicular feminisation (Morris, 1953; Morris & Mahesh, 1963) which is due to androgen insensitivity (Southren, 1965; Strickland & French, 1969; Wilkins, 1957), the patient is a 46,XY genotypic male with normally functioning testes.

Testosterone is produced (Tremblay et al., 1972) but there is end-organ insensitivity to testosterone and dihydrotestosterone; therefore the Wolffian duct is not stabilised and hence it regresses. The urogenital sinus does not respond to androgens, therefore lack of male development results in female external genitalia. Externally an almost normal phenotypic female results. The Mullerian inhibitory factor is produced normally by the foetal testis, and because this is not an androgen (Jost, 1953) the end-organ (Mullerian duct) responds normally by regressing. This results in a blind-ending vagina: the uterus and fallopian tubes are not present. The descent of the testes is also controlled by androgens, therefore in testicular feminisation, intra abdominal or inguinal testes are found. This syndrome accentuates the fact that androgens are required for the development of male external genitalia and internal genital ducts, and also demonstrates that MIF is not an androgen. However, it gives no clue to the nature of MIF, and does not disprove the postulate that progesterone is involved in Mullerian inhibition. In fact, sex steroids other than androgens are still effective in these patients as evidenced by breast development in response to testicular oestrogens.

In the rare condition hernia uteri inguinalis in the male (Young, 1951) genotypic males only have a single testis. This solitary testis functions normally as an endocrine gland and a generative organ. There is unilateral (ipsilateral) inhibition of the Mullerian duct and stimulation of the Wolffian duct. The external genitalia, which develop under the influence of systemic dihydrotestosterone and not to locally-acting testosterone, develop as normal male organs. The contralateral sex ducts, however, follow female developmental lines with formation

of a uterus and fallopian tube. This syndrome illustrates the local, ipsilateral, action of foetal testicular secretions on the genital ducts, but does not indicate the nature of MIF nor does it disprove the progesterone postulate.

The most common cause of intersexuality is pseudohermaphroditism due to congenital adrenal hyperplasia (Bongiovanni & Root, 1963a,b & c). Steroidogenic enzymes in the adrenal cortex and testis are lacking with resultant deficiency of glucocorticoid secretion. The negative feedback inhibition of the hypothalamus and pituitary is therefore reduced resulting in over-secretion of corticotrophin. Adrenal androgens, which are usually produced in relatively small amounts, become a major secretory product causing virilisation.

The most common defects in congenital adrenal hyperplasia are deficiencies of 21 hydroxylase and 11β-hydroxylase, neither of which are required for androgen biosynthesis (Fig. 1.3). These deficiencies, therefore, do not affect testicular steroidogenesis.

More serious forms of congenital adrenal hyperplasia involve deficiencies of cholesterol 20:22 desmolase (Prader & Liebenmann, 1957) and 3β-HSD-I (Bongiovanni & Kellenbenz,1962), the former causing impaired synthesis of all active steroid hormones. Total lack of cholesterol 20:22 desmolase is incompatible with life (Bongiovanni et al., 1957); and partial deficiency which requires compensatory hyperactivity of steroid-producing organs does not preclude the secretion of progesterone by the foetal testis.

Deficiency of 3β-HSD poses an interesting problem. Not only are glucocorticoids lacking but testosterone synthesis is also affected. Excessive stimulation of the adrenal cortex results in secretion of large amounts of DHA, a steroid with

androgenic properties, but not as androgenically active as testosterone. Therefore, in males there is androgen deficiency whereas in females there is excessive androgen secretion. As a result, male foetuses are feminised because of lack of androgens, and female foetuses are virilised due to over production of androgens (Goldman et al., 1964). If Mullerian inhibition requires progesterone, deficiency of 3β-HSD should results in persistence of the Mullerian ducts. Not many patients with this rare form of congenital adrenal hyperplasia have been described. If the defect is incomplete, some progesterone may be secreted. Complete deficiency of 3β-HSD is rapidly fatal (Bongiovanni & Kellenbenz, 1962), and in such cases a complete description of the internal genitalia in association with karyotype has not been published.

The condition of congenital adrenal hyperplasia, therefore, does not give any indication as to the nature of MIF nor does it discount the postulate that progesterone is involved. Of interest, though, is the presence of varying degrees of Mullerian duct development in male patients with 3β-HSD deficiency (Goldman et al., 1964; Bongiovanni & Kellenbenz, 1962): this suggests that steroidogenesis is involved to some extent in Mullerian inhibition.

Congenital adrenal hyperplasia due to deficiency of 17α-hydroxylase (New, 1970) of C^{17-20} desmolase (Zachmann et al., 1972) cause feminisation of male foetuses because of lack of testosterone secretion. In both of these syndromes, MIF is produced normally, but as neither enzyme is required for progesterone synthesis it is possible that progesterone is involved.

Intersexuality due to true hermaphroditism is rare and complex, and does not help in the determination of the nature of MIF.

On theoretical grounds it was postulated that progesterone may be required for inhibition of the foetal Mullerian duct. Progesterone has an inhibitory effect on the Mullerian duct in organ culture, and although it shows some cytoxic effects, it appears to inhibit the Mullerian duct specifically, and is capable of stabilising the Wolffian duct in vitro. Foetal testicular cells in monolayer culture produce and release progesterone and in the process of maturation, progesterone is secreted before testosterone biosynthesis is demonstrable. Clinical syndromes characterised by abnormal sexual development do not contradict the progesterone hypothesis.

The evidence presented in this thesis is circumstantial and inconclusive. However, I believe there are grounds to pursue the possibility that progesterone and MIF are related. Genital tracts complete with testes attached should be cultured with a progesterone antagonist (for example anti-progesterone serum), or with an inhibitory of progesterone biosynthesis (eg. cyanoketone) The binding of progesterone to androgen binding protein should be examined, and the effect of an ABP-progesterone complex on the Mullerian duct should be studied. Foetal calf testes in organ culture produce MIF which can be concentrated: further characterisation of this preparation is required.

Since the pioneer work of Jost in 1947, many workers have attempted to characterise MIF. Much work has been done, but we have progressed little. Mullerian inhibition is fundamental to sexual differentiation, and the elucidation of its nature warrants further study.

SECTION II : HUMAN TUMOURS

CHAPTER 5

INTRODUCTION

CHAPTER 5

INTRODUCTION

A. ALPHA$_1$-FETOPROTEIN AND TESTICULAR TUMOURS

Bergstrand and Czar (1956) first described an α_1-globulin in the serum of human foetuses. This globulin is now known as alpha$_1$-fetoprotein (AFP) and is one of the main serum proteins in early embryonic life in many animal species (Gitlin and Boesman, 1966,1967). It is produced mainly by the yolk sac and the foetal liver (Gitlin et al., 1967) but small quantities are also produced by the gastrointestinal tract, kidney and placenta (Gitlin et al., 1972). The foetal shark stomach is a major site of AFP production (Gitlin, 1971,1974). In the human foetus, a peak serum AFP level of 3mg/ml occurs at 13 weeks gestation (Gitlin & Boesman, 1966) and this value falls to 10-150µg/ml at delivery (Norgaard-Pedersen, 1976), the final value depending on gestational age at delivery (Sananes et al., 1973). The normal adult range of 4-10.5ng/ml (Ruoslahti & Seppala, 1972) is reached by the age of 2 years (Masseyeff et al., 1975) and this value normally remains consistent till after the age of 60 years when a further slight fall in serum value occurs (Masseyeff et al., 1974).

Abelev et al. (1963) demonstrated that an α-globulin present in the serum of foetal mice reappears in the serum of adult mice bearing a transplantable hepatoma which was originally induced by a chemical carcinogen. Non-neoplastic liver regeneration following partial hepatectomy also results in reappearance of the foetal α_1 -globulin in the serum of adult

mice. Tatarinov et al. (1964,1965) were the first to demonstrate a foetal globulin in the serum of humans with primary hepatomas; but cholangiocarcinomas and metastatic carcinomas in the liver did not apparently produce this globulin (Tartarinov et al., 1967). The foetal globulin appearing in the serum of hepatoma-bearing mice and humans is AFP. Abelev et al. (1967) confirmed the presence of AFP in the serum of hepatoma patients, and also noted its presence in the serum of some patients with testicular tumours: in Abelev's series, patients with pure testicular seminoma or pure testicular choriocarcinoma did not have elevated serum AFP, nor did patients with sarcoma arising from the non-germinal stromal elements of the testis (Abelev, 1968; Abelev et al., 1967). Since then, I, (Grigor et al., 1977) and many other workers, have demonstrated AFP production by testicular teratomas (Table.5.I).

AFP levels are now known to be elevated in body fluids in association with many neoplastic and non-neoplastic conditions (see review by Neville & Cooper, 1976). Measurement of AFP levels in amniotic fluid is used extensively in obstetric practice for the prenatal diagnosis of neural tube defects, anencephaly, hydrocephalus, congenital oesophageal atresia, congenital nephrosis, exomphalos, tetralogy of Fallot, foetal sacrococcygeal tumour and intra-uterine death (Norgaard-Pedersen, 1976; Chaube and Swinyard, 1975). Maternal serum AFP is elevated during pregnancy, but higher than normal values are associated with foetal distress, intrauterine death, twin pregnancy, toxaemia, rhesus isoimmunisation, and various foetal congenital abnormalities (Norgaard-Pedersen, 1976). Ferguson-Smith et al.(1978) have shown that the vast majority of anencephalic and spina

Table 5.I. Incidence of raised serum AFP associated with testicular tumours and male germ cell tumours reported by various authors.

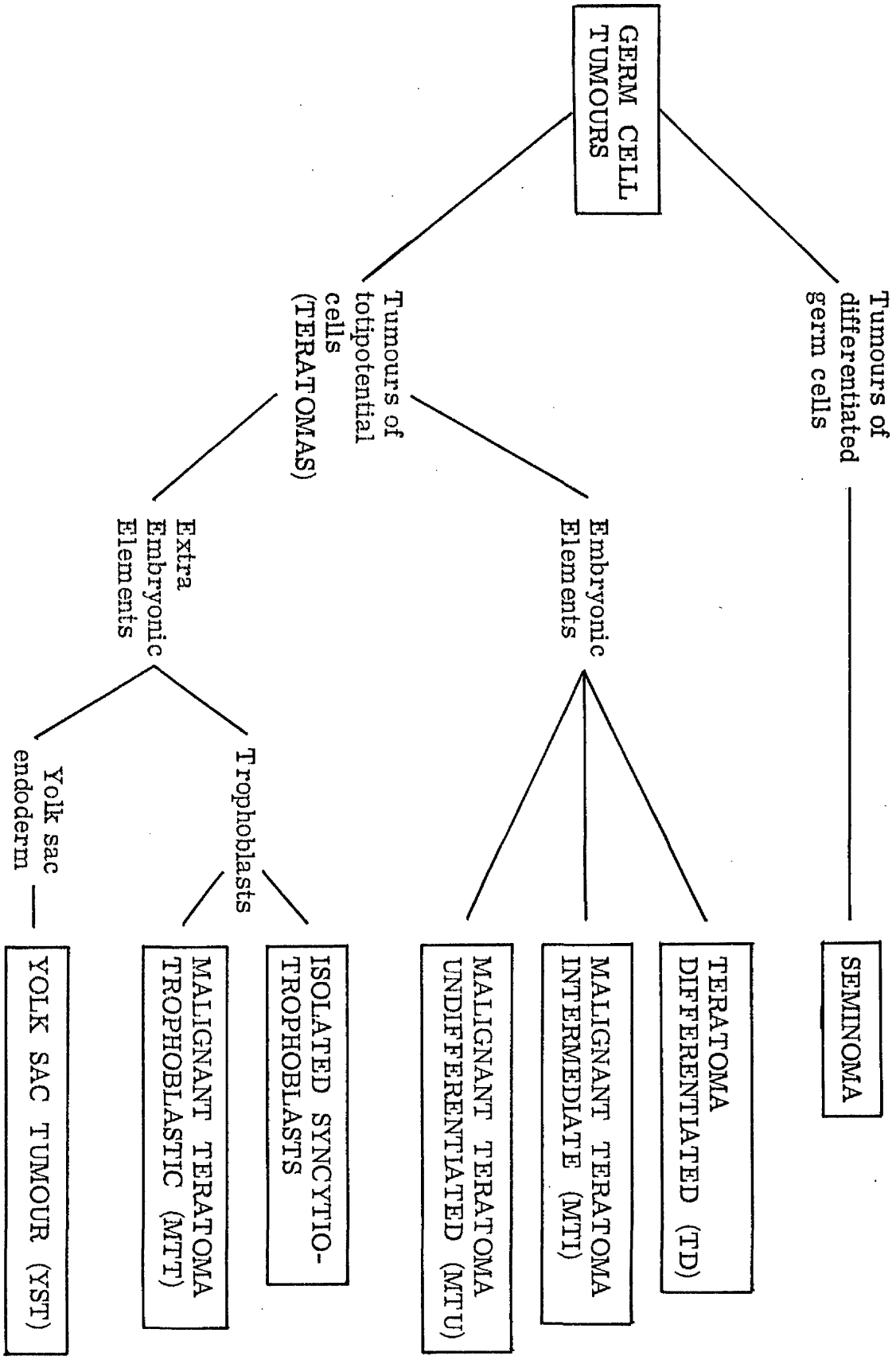
Author	Number of patients	Type of tumour	Number of patients with raised serum AFP	Raised serum AFP
Abelev (1968)	32	teratoblastomas	13	41 %
Abelev et al. (1971)	32	teratoblastomas	15	68 %
Ballas (1974)	6	embryonal carcinoma	3	50 %
Bracken et al. (1975)	50	active teratocarcinoma or embryonal carcinoma	15	50 %
Buffe (1973)	37	active teratomas of children	27	72 %
Elgort et al. (1973)	21	testicular terotoblastomas	19	91 %
Grigor et al. (1977)	88	active teratomas	56	67 %
Lange et al. (1976)	30	preoperative teratomas	23	77 %
Mawas et al. (1971)	27	active malignant teratomas in children	15	71 %
Merrin et al. (1971)	14	active teratomas	3	21 %
Newlands (1976)	44	teratomas	38	86 %
Norgaard-Petersen et al. (1975)	19	germ cell tumours with yolk sac tumour	19	100 %
Perlin et al. (1976)	9	male germinal tumours	5	56 %
Talerman et al. (1974)	30	active germ cell tumours	8	27 %
Talerman et al. (1977)	7	male germ cell tumours with yolk sac tumour	7	100 %
Tsuchida et al. (1973)	3	yolk sac tumours	3	100 %

bifida births can be avoided by screening maternal serum for AFP at 16-20 weeks gestation. Raised serum AFP may be associated with malignant diseases other than tumours of the liver or germ cell tumours, especially tumours of the gastro-intestinal tract, particularly if liver metastases are present (McIntire et al., 1975), but in such cases, serum CEA is a better tumour marker (Grigor et al., 1975). Serum AFP may be elevated in patients with acute and chronic liver disease and this is probably a reflection of hepatic regeneration (Eleftherion et al., 1977; Silver et al., 1974a&b). Patients with ataxia telangiectasia (Waldmann & McIntire, 1972) or cystic fibrosis (Chadra et al., 1975) have moderately raised serum AFP levels.

The present study is concerned with male patients with gonadal or extragonadal germ cell tumours, and the correlation between serum levels of AFP, histological features of the tumours and the clinical course of the disease. In some patients, serum or urinary human chorionicgonadotrophin was also measured.

Many previous attempts at correlating serum AFP with the histological patterns of testicular tumours have been hampered by the fact that two major classifications of testicular tumours (Mostofi & Price, 1973; Pugh, 1976) fail to stress the importance of recognising within teratomas areas of yolk sac (endodermal sinus) differentiation as described by Teilum (1959). In this thesis, germ cell tumours are classified according to the British Testicular Tumour Panel and Registry (Pugh, 1976) with minor modifications so that the significance of extraembryonic elements can be assessed (Fig.5.1). The criteria used in this thesis for diagnosing yolk sac (endodermal sinus) tumour are those of Teilum (1976), and

Fig. 5.1. Classification of germ cell tumours of the testis and extra-gonadal sites.



the histological features of yolk sac tumour (YST) are shown in Fig. 6.4. Yolk sac elements are found in patients of all ages although Pugh (1976) maintains that YST only occurs in infant testicular tumours.

In 1967 Abelev suggested that AFP production by testicular "teratoblastomas" may be due to cells destined to become hepatoblasts. Gitlin et al.(1967) and Gitlin and Boesman (1966) demonstrated that the yolk sac is an important source of AFP in the foetal circulation. Teilum (1959) previously suggested that certain histological patterns in testicular and ovarian germ cell tumours should be regarded as yolk sac (endodermal sinus) differentiation because of similarities with the rat placenta. Abelev (1971) therefore suggested that AFP production by testicular tumours is a result of yolk sac differentiation. Laurence & Neville (1972) stated that if the endodermal sinus tumour is of vitelline origin, as proposed by Teilum (1971), then it should produce AFP. Talerman & Haije (1974) supported this contention, and many reports since then have shown a strong correlation between AFP production and the presence of yolk sac elements in testicular tumours (Ballas, 1974; Grigor etal., 1977; Norgaard-Pedersen, 1976; Norgaard-Pedersen et al., 1975,1976; Sell et al., 1976; Talerman et al., 1977; Teilum et al., 1975; Tsuchida et al., 1973). There are also many studies demonstrating AFP in yolk sac structure by immunocytochemical techniques (Ito et al., 1976; Palmer et al., 1976; Shirai et al., 1976; Teilum et al., 1974; Yoshiki et al., 1976).

It has been claimed that AFP is a specific marker for yolk sac elements in testicular tumours (Norgaard-Pedersen et al., 1975; Teilum et al., 1975; Sell et al., 1976a; Talerman et al., 1977b) but the correlation

is not always absolute (Ballas, 1974; Grigor et al., 1977). Abelev (1974), basing his conclusions on immunofluorescence studies in mouse teratomas (Englehardt et al., 1973), has suggested that other endodermally derived epithelial elements may produce AFP.

Human chorionicgonadotrophin (HCG) is a glycoprotein hormone produced by the syncytiotrophoblasts of the placenta throughout pregnancy, being first detectable at 9-10 days after conception (Keller, 1976). It consists of α and β subunits. The α subunit of HCG is similar to the α subunits of luteinising hormone (LH) follicle stimulating hormone (FSH) and thyroid stimulating hormone (TSH); but the β subunit is specific for HCG (Vaitukaitis & Ross, 1974). Antiserum to β HCG specifically detects HCG even in the presence of other glycoprotein trophic hormones (Vaitukaitis et al., 1972). Serial HCG determination is of proven value in the monitering of gestational choriocarcinomas (Bagshawe, 1974). Using specific antisera to the β subunit, it has been shown that testicular tumours also produce HCG (Vaitukaitis, 1974). However, many other non-trophoblastic tumours are also associated with HCG synthesis and release (Braunstein, 1973b; Goldstein et al., 1974; McManus et al., 1976).

Pugh states that the presence of syncytiotrophoblasts and cytotrophoblasts arranged in a papillary or villous pattern is essential for the diagnosis of malignant teratoma trophoblastic (MTT). In such tumours, other teratomatous elements are of minor prognostic significance and need not be mentioned in the classification (Pugh, 1976). Mostofi & Price (1973) and Teilum (1976) do not insist on the papillary arrangement of the syncytio- and cytotrophoblasts in order to make a diagnosis of choriocarcinoma

of the testis, and these authors differentiate between pure choriocarcinoma and choriocarcinoma associated with other germ cell derivatives. In the present series, MTT indicates the presence of syncytiotrophoblasts and cytotrophoblasts arranged together, but not necessarily forming a papillary pattern: the presence of any other associated tumour elements was also recorded. In addition, isolated tumour giant cells which contained βHCG as demonstrated by immunocytochemical techniques (Heyderman & Neville, 1976a&b) were classified as isolated syncytiotrophoblasts.

In the present study, male patients with testicular and extragonadal germ cell tumours were examined serologically for evidence of AFP production, and the histological features of the tumours were re-assessed. The prognostic significance of elevated serum AFP and the presence of various histological patterns will be presented (Chapter 6). Details of 153 of these patients have already been published (Grigor et al., 1977) and this is,at present, the largest published series correlating serum AFP with histology of testicular tumours and clinical course. The strong correlation between AFP production and the presence of yolk sac elements is substantiated in this paper, in agreement with many other authors. However, the lack of absolute correlation between these two features was mentioned and this is an aspect of AFP production that is not well recognised. I have now examined a much larger series of patients, and this thesis presents data which is not available from other centres because of lack of numbers of patients.

B. NON-HORMONAL ADRENOCORTICAL CARCINOMAS

Adrenocortical carcinomas are rare tumours which may be classified as "functioning" or"non-hormonal" according to the clinical syndromes with which they are associated. Hypercorticalism takes the form of the adrenogenital syndrome (virilation or feminisation), Conn's syndrome (hyperaldrosteronism with low plasma renin), or Cushing's syndrome (Neville and MacKay, 1972; Neville and Symington, 1966, 1967; Symington, 1969). Adrenocortical tumours are considered to be "non-hormonal" if there is no clinical evidence of endocrine upset. They are very rare; wide experience is difficult to obtain at one institution, as is exemplified by the large number of single case reports in the literature over the last 30 years.

An analysis of these cases along with 20 additional new cases seen at the Royal Marsden Hospital, London, Royal Infirmary, Glasgow, and the Institute of Cancer Research, London, was undertaken in order to trace some aspects of their behaviour and response to treatment. The findings of this survey have already been published (Lewinsky et al., 1974) and will be presented as part of the present thesis in Chapter 7 as a further example of correlation - or lack of correlation - between morphology and function.

SECTION II : HUMAN TUMOURS

CHAPTER 6

TESTICULAR TUMOURS

CHAPTER 6

TESTICULAR TUMOURS

The aim of this part of the thesis is to attempt to explain why patients with teratomas sometimes have elevated serum AFP levels. In particular, I have tried to ascertain which cell type is responsible for AFP production. It is well known that endodermal sinus tumours of the ovary carry a very bad prognosis (Beilby & Parkinson, 1975; Beilby & Todd, 1974): I have examined similar tumours in male patients to determine if such tumours have the same grave prognosis in both sexes.

A. MATERIALS AND METHODS

1. Patients

All patients were referred to the Royal Marsden Hospital (RMH), Sutton and London branches, for treatment of testicular or extragonadal germ cell tumours. There were 322 patients all of whom had histological material of their tumour available for review, and had serum assayed for AFP. Of these, 153 patients have already been described (Grigor et al., 1977) and have now been followed for at least 2½ years in order to study features of prognostic significance. A further 169 patients have been added to this group in order to increase the number available for morphological and functional correlation, but this latter group have not been followed for a sufficient length of time to make prognostic assessments.

2. Histological material

Twenty-four patients had inguinal orchidectomy with high ligation of the spermatic cord performed at RMH.

Several blocks of tumour, adjacent testis, and cord were examined histologically. However, the majority of patients had initial surgery at other hospitals before referral to RMH, and surgical and pathological practices often differed. In many instances, a limited number of histological sections were avilable for review, therefore some histological features of relevance may have been missed because of incomplete sampling.

In this seris, 316 patients had testicular or extragonadal germ cell tumours, and 6 patients had non-germ cell tumours of the testis.

3. Age distribution

The age distribution of first diagnosis of 316 male patients with testicular or extragonadal germ cell tumours is shown in Fig. 6.1. The peak incidence of teratomas is in the 3rd decade, and that of seminomas extends over the 3rd, 4th and 5th decades. Yolk sac elements, and trophoblastic elements (including isolated syncytiotrophoblasts) are present in germ cell tumours of all ages (Figs. 6.2 and 6.3) and the proportion of teratomas containing extraembryonic elements is relatively constant throughout the age spectrum. Figure 6.2 shows that the proportion of teratomas containing yolk sac elements appears to be greater at both extremes of the age spectrum. However, such a conclusion cannot be made with certainty because of the small number of patients in these age groups.

4. Site

The distribution of primary site of 316 germ cell tumours in males is shown in Table 6.I.

Fig. 6.1. Age distribution at first diagnosis of 316 male patients with testicular and extragonadal germ cell tumours.

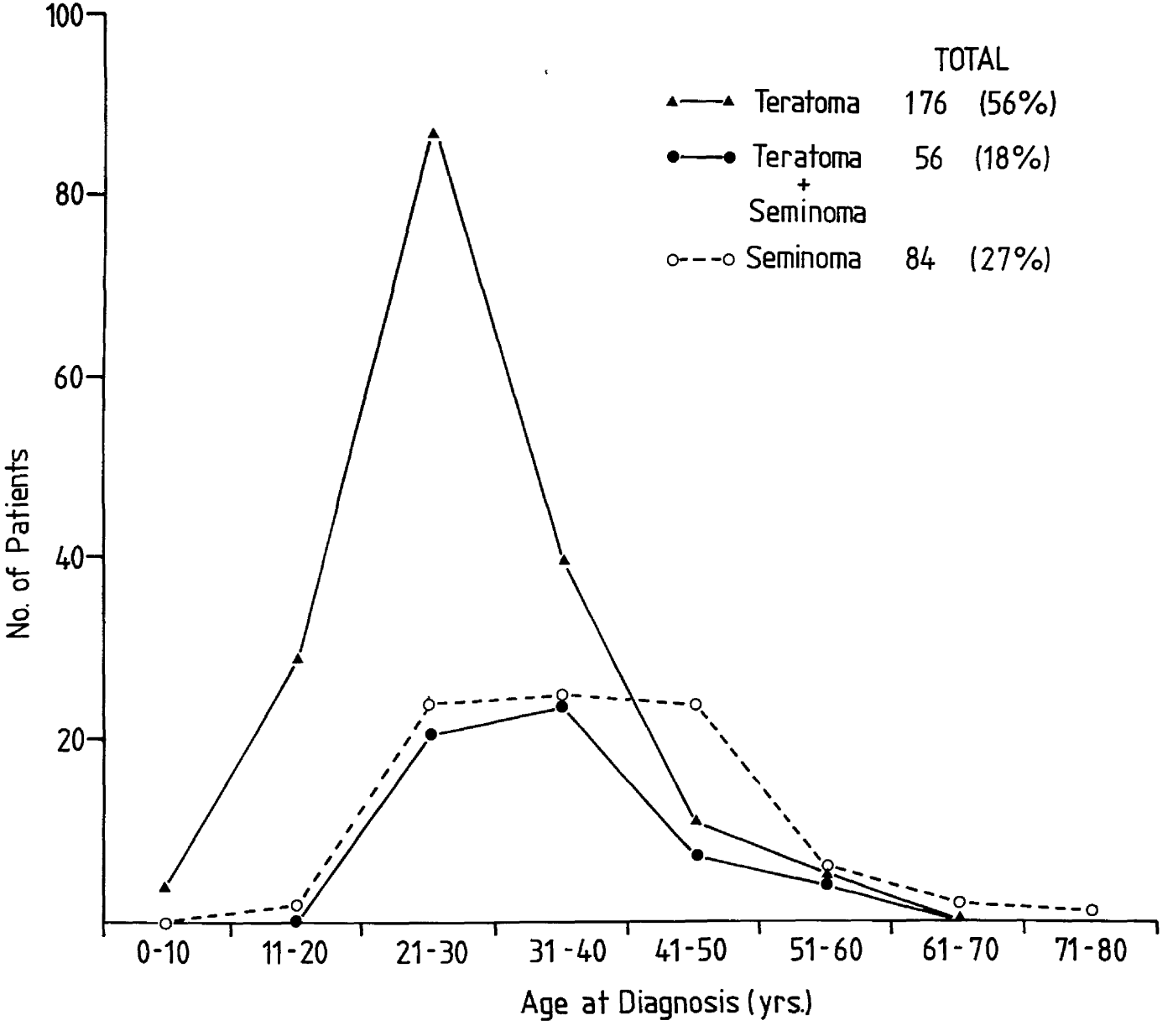

100
80
60
40
20
No. of Patients
TOTAL
Teratoma 176 (56%)
Teratoma + Seminoma 56 (18%)
Seminoma 84 (27%)
0-10
11-20
21-30
31-40
41-50
51-60
61-70
71-80
Age at Diagnosis (yrs.)

Fig. 6.1.

Fig. 6.2. Age distribution of 232 patients with teratomas (± seminoma) and the proportion of teratomas containing yolk sac elements either alone, or in combination with other teratomatous elements.

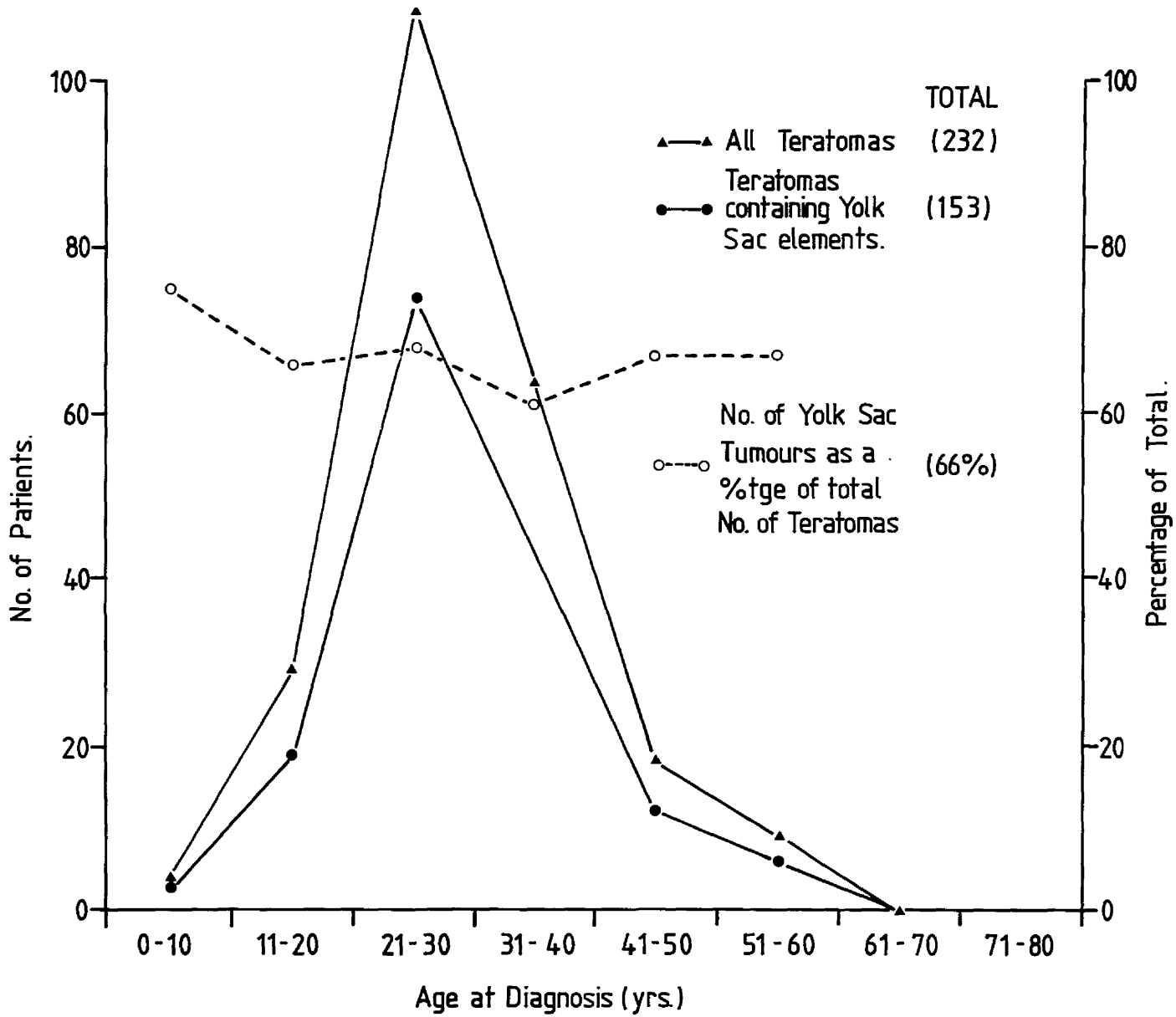

TOTAL
All Teratomas (232)
Teratomas containing Yolk Sac elements. (153)
No. of Yolk Sac Tumours as a %tge of total No. of Teratomas (66%)
No. of Patients.
Percentage of Total.
100
80
60
40
20
0
0-10
11-20
21-30
31-40
41-50
51-60
61-70
71-80
Age at Diagnosis (yrs.)

Fig. 6.2.

Fig. 6.3. Age distribution of 232 patients with teratomas (± seminoma) and the proportion of teratomas containing trophoblastic elements in the form of MTT or isolated syncytiotrophoblasts.

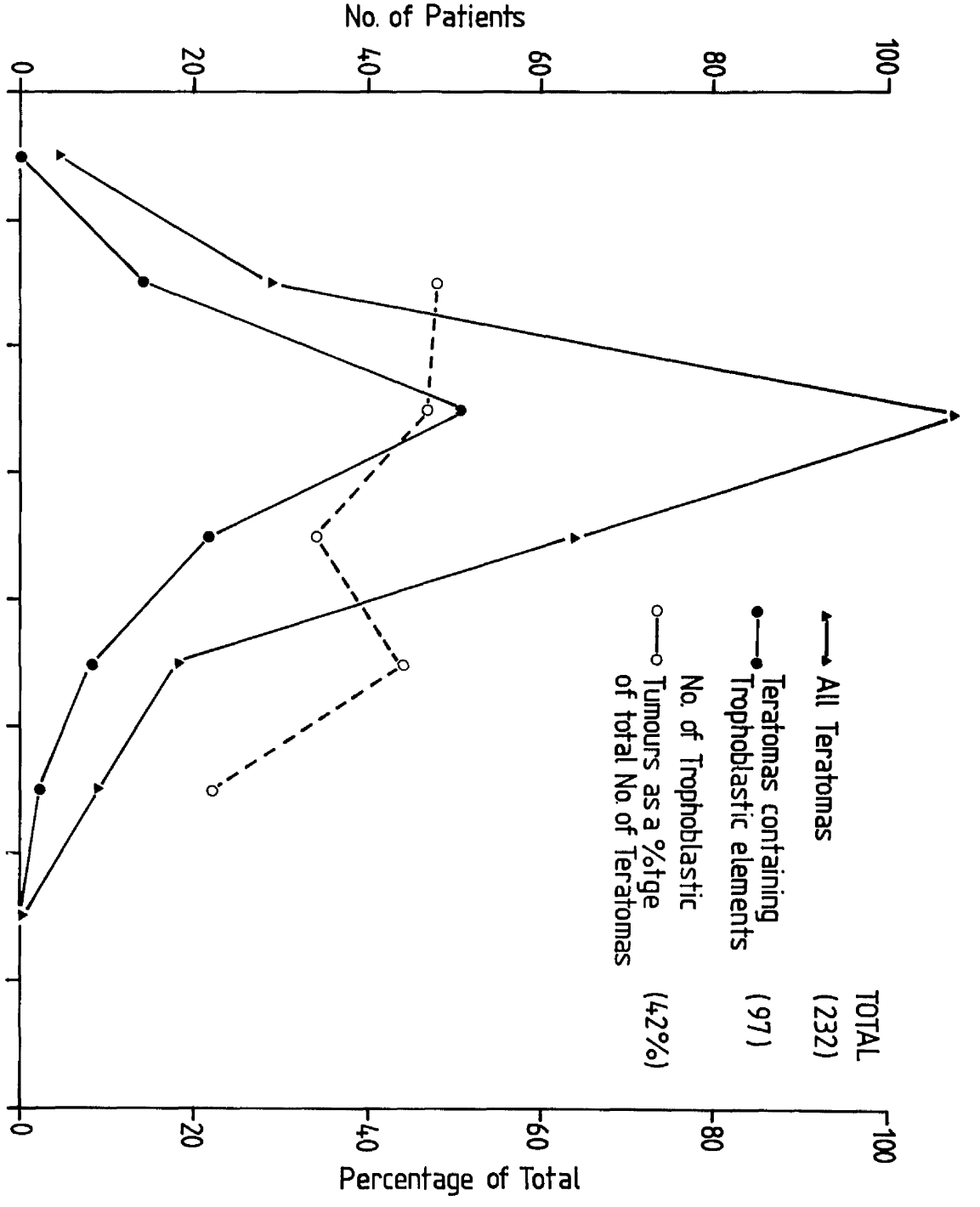

No. of Patients
0
20
40
60
80
100
0-10
11-20
21-30
31-40
41-50
51-60
61-70
71-80
Age at Diagnosis (yrs.)
Percentage of Total
All Teratomas
TOTAL (232)
Teratomas containing Trophoblastic elements
(97)
No. of Trophoblastic Tumours as a %tge of total No. of Teratomas
(42%)

Fig 6.3.

Table 6.I. Primary site of 316 germ cell tumours in male patients.

TESTICULAR	
left testis	137
right testis	143
bilateral	13
not stated	9
subtotal	302
EXTRAGONADAL	
retroperitoneal	8
mediastinal	5
unknown	1
subtotal	14
TOTAL	316

5. Clinical staging of disease

All patients admitted to the Royal Marsden Hospital were examined thoroughly for the extent of tumour spread at presentation (Table 6.II). Lymphangiography (LG) and chest X-ray (CXR) were standard procedures, and intravenous pyelography (IVP), lung tomography and ultrasonography (US) were used when indicated. Towards the end of the survey, computerised axial tomography (CAT) using an EMI scanner was available. Retroperitoneal lymphadenectomy as a staging procedure was not routine. On this basis, patients were staged as follows:-

Stage 1 : Tumour confined to one, or both, testes

Stage 11 : Metastases to lymph nodes below the diaphragm

Stage 111 : Metastases to lymph nodes above the diaphragm

Stage 1V : Extranodal metastases

The clinical staging of 232 patients with teratoma or combined tumour is shown in Table 6.III. Of these, 60 patients (26%) presented with tumour confined to the testis and 172 (74%) had metastatic disease.

6. Serum samples

Ten ml of venous blood was collected and the serum separated by centrifugation within 3 hours and stored at $-70^{o}C$. Only 5 preorchidectomy serum samples were available for study because of the referral system used, but a sample was taken when the patient first attended RMH before commencement of post-operative therapy, and at each subsequent follow-up visit.

7. Alpha-fetoprotein (AFP) assay

Serum AFP was measured by radioimmunoassay. Anti-AFP serum was raised in rabbits, and the free and bound fractions separated by precipitation with ammonium sulphate or ethylene glycol.

Table 6. II. Ancillary staging procedures used in addition to full clinical examination for patients with testicular and extragonadal germ cell tumours.

Investigation	Site of metastases examined
chest X - ray tomography	lung mediastinal lymph nodes chest wall
lymphangiography (standard procedure)	abdominal (para-aortic) lymph nodes mediastinal lymph nodes
lymphangiography (specialised procedure)	renal hilar lymph nodes
ultrasound	liver abdominal
scanning (isotope scintillography)	bones liver brain lung
computerised axial tomography (CAT) (EMI scan)	brain abdomen any other site

Table 6.III. Clinical staging of 232 male patients with teratoma (± seminoma) according to main histological type.

Main histological type	Clinical stage				Total
	I	II	III	IV	
malignant teratoma undifferentiated (MTU)	14	9	3	66	92
malignant teratoma intermediate (MTI)	34	21	9	29	93
teratoma differentiated (TD)	4	0	2	2	8
malignant teratoma trophoblastic (MTT)	0	0	0	4	4
yolk sac tumour (YST)	8	2	3	22	35
Total	60	32	17	123	232

The sensitivity of this method is 2 ng AFP per ml of undiluted serum. Serum samples from 32 adult males (aged 20-40 years) with no evidence of hepatic or malignant disease had AFP levels between 2 and 16 ng/ml serum (mean: 4.6± 2.6 ng/ml). Values in excess of 25 ng/ml were considered in this study to be elevated, and values between 16 and 25 ng/ml were equivocal but not definitely raised. The prime purpose of the assay was to detect and quantitate elevated serum AFP rather than to study fluctuations in the normal range. Therefore, for many of the samples, the lower limit of sensitivity of the assay was set at 25 ng/ml so that the working range of the assay was extended at the upper level.

B. RESULTS

1. Serum AFP levels as a function of type and activity of the tumour

The 322 patients in this survey were classified according to their main histological type, viz. teratoma; seminoma; combined teratoma and seminoma; and non-germ cell testicular tumour. All had serum AFP measured while attended the Royal Marsden Hospital but 125 patients had no evidence of residual tumour by the time they were first seen at RMH, and no evidence of recurrence was seen in the follow-up period. These 125 patients were considered to have "non-active" disease. All other patients had "active disease" at some time in the course of serum AFP determination although the tumour was not always clinically apparent. The incidence of raised serum AFP (>25 ng/ml) is shown in Table 6.1V.

A total of 120 patients had raised levels, and 110 of these had active teratoma (± seminoma) at the time of serum sampling. Ten patients had elevated serum AFP at a time when there was apparently no active teratoma present, and details of these patients are shown in Table 6.V. Four of these patients (1,2,6 & 10) had only a slight transient elevation of serum AFP and this was seen in only one serum sample: previous and subsequent samples were normal. These isolated elevations may have been caused by non-specific ailments, for example mild liver dysfunction. Another 4 patients (3,4,7 & 8) had serum samples taken shortly after orchidectomy and AFP in the serum may not have had time to fall to normal levels. In such cases, a preoperative sample would have been very useful.

Table 6.IV. Serum AFP in 322 patients with germ cell tumours and non-germ cell testicular tumours according to main histological classes and clinical activity of the tumour.

Pathology	Total number of patients	Activity of disease *	Serum AFP **	
			Normal	Raised
Seminoma	84	Active	32	1
		Non active	51	0
Seminoma + Teratoma	51	Active	13	17
		Non active	19	2
Teratoma	181	Active	39	93
		Non active	43	6
Non-germ cell tumour of testis	6	Active	1	1
		Non active	4	0
Total	322		202	120

* Active = Serum sample taken at a time when viable tumour was present in the body

Non active = No clinical evidence of active tumour during period of serum sampling and no evidence of tumour recurrence throughout the follow-up period

** Normal range of serum AFP :- less than 25 ng/ml

Table 6.V. Clinical details of patients who had raised serum AFP levels without apparent active teratoma : none of these patients developed evidence of recurrent or residual teratoma in the remainder of the period of investigation.

Patient	Serum AFP (ng/ml)	Clinical status at sampling	Comments
1 (B.B.)	32 (on a single occasion)	active seminoma in para-aortic nodes	only 1 slide of original tumour available. Serum sample taken 3 days after laparotomy
2 (G.W.)	29 (on a single occasion)	active paratesticular rhabdomyosarcoma. lung metastases	
3 (D.M.)	100	9 days after orchidectomy for stage I tumour	tumour contained MTI + YST + seminoma
4 (J.V.)	340	11 days after orchidectomy for stage I tumour	tumour contained YST + seminoma
5 (M.S.)	52	no evidence of residual tumour : infant aged $1\frac{1}{2}$ yrs	pure YST stage I removed 6 months previously
6 (C.B.)	36 (on a single occasion)	no evidence of residual tumour	MTU stage I removed 6 weeks previously
7 (J.F.)	96	12 days after orchidectomy for stage I tumour	tumour contained MTI + YST
8 (M.M.)	160	3 days after orchidectomy for stage I tumour	tumour contained MTI + YST
9 (S.W.)	49	13 days after orchidectomy for stage I tumour	11 month old infant tumour was TD
10 (R.L.)	28 (on a single occasion)	10 weeks after orchidectomy for stage I tumour	tumour contained MTI

Patients 5 & 9 were infants and the mildly elevated serum AFP levels may have been physiological.

If we exclude (a) small elevations seen in infants, (b) small transient elevations in adults which cannot be verified by a repeat serum sample; and (c) postoperative samples before sufficient time has elapsed for high pre-operative levels to fall to normal, then in the present series, a confirmed elevated serum AFP always indicated the presence of active teratoma.

2. Histological sub-types and serum AFP levels

Fifty-four patients had active teratomas consisting of purely embryonic elements (TD,MTI, MTU) or purely one type of extra embryonic component (YST or MTT): some of these tumours also contained seminomatous elements but such elements were considered non-contributory to serum AFP. Table 6.VI. shows the correlation between teratomas of pure sub-type and serum AFP levels. The patient with TD was an infant with a single minimal elevation of serum AFP (Table 6.V: patient No.9) which may not have been related to the tumour (vide supra). Pure trophoblastic tumours are not associated with raised serum AFP, and pure YST is most consistently accompanied by raised levels.

3. Incidence of yolk sac tumour (YST) in teratomas

The presence or absence of YST, as described by Teilum (1976) and illustrated in Fig. 6.4., in 232 teratomas is shown in Table 6.VII. Yolk sac elements appeared in teratomas in all age groups (Fig. 6.2), and the percentage of teratomas containing YST in different age groups remains fairly constant (61-75%). YST may occur in pure extra-embryonic teratomas, or in combination with embryonic elements (Table 6.VIII).

Table 6. VI. Relationship of specific histological features to serum levels of AFP in 54 patients who had active teratoma of a single histological sub-type.

Serum AFP *	Teratomas consisting entirely of				
	TD	MTI	MTU	MTT	YST
normal	0	5	8	4	2
elevated	1	1	17	0	16

* Normal range of serum AFP : less than 25 ng/ml

Table 6. VII. Incidence of yolk sac elements in teratomas.

Yolk sac elements	Number of teratomas	Incidence
present	153	66 %
absent	79	34 %
Total	232	100 %

Fig. 6.4. Histological patterns seen in yolk sac tumours.

Fig. 6.4a. Microcystic pattern. Flattened endothelial cells form a reticular microcystic network having a honeycomb appearance. Intracellular and extracellular eosinophilic hyaline globules are present: this material stains with periodic acid schiff (PAS) and is diastase-resistant. Immunocytochemical staining has located AFP in these globules. This pattern of YST is common in the infantile testes. (H & E X 180)
Testicular tumour in male aged 3½ months. Pure YST.

Fig. 6.4b. Macrocystic pattern. In some areas, the cystic spaces are much larger, but the lining cells have a similar morphology to those of Fig. 6.4b. (H & E X 108)
Testicular tumour in male aged 38 yrs. YST + MTI.

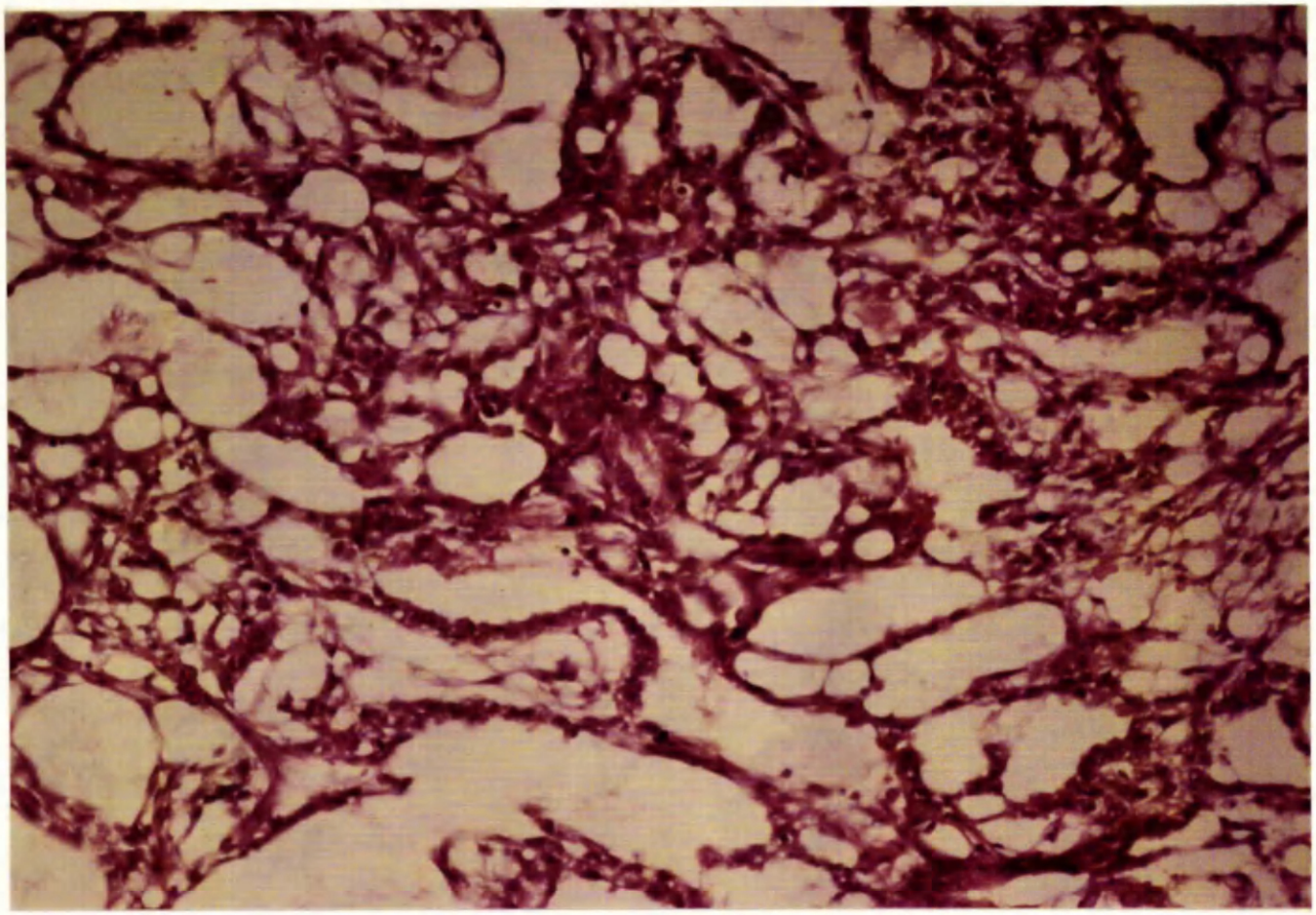


Fig. 6.4a.

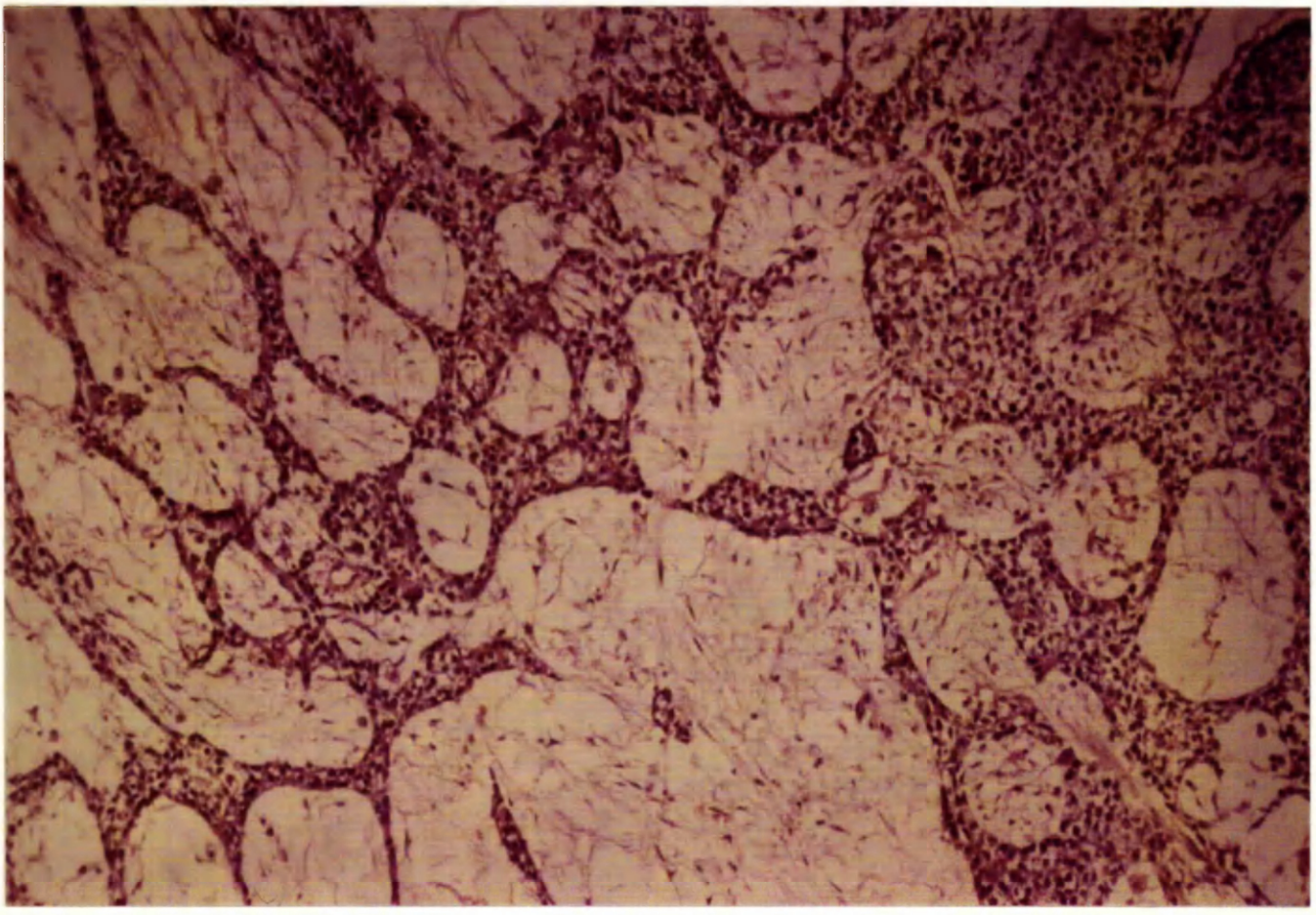


Fig. 6.4b.

Fig. 6.4. Histological patterns seen in yolk sac tumours.

Fig. 6.4c. Endodermal sinus body. This structure, also known as a "Schiller Duval" body, is pathognomonic of YST. It consists of a mantle of cuboidal endothelial cells surrounding a fibrovascular core, and the central structure is surrounded by an outer layer of similar cuboidal cells.

(H & E X 450)

Testicular tumour in male aged 34 yrs. YST + MTU.

Fig. 6.4d. Polyvesicular vitelline component. This pattern is frequently present in ovarian YST, but is also noted in some testicular tumours. It consists of blastocyst-like vesicles lined by flattened or cuboidal mesothelial cells in a cellular compact stroma. (H & E X 180)

Ovarian tumour: this slide was kindly donated by Prof. G. Teilum.

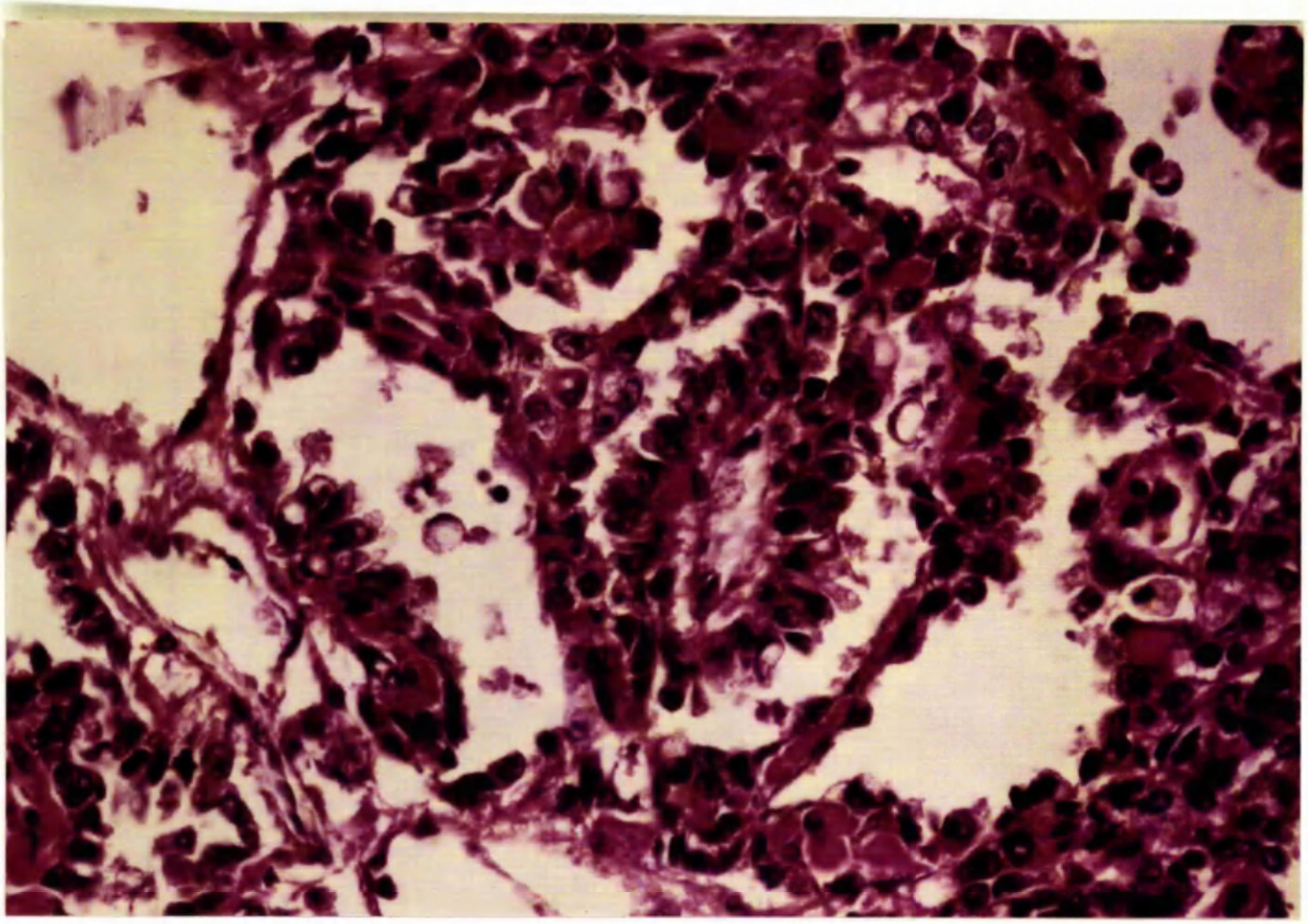


Fig. 6.4c.

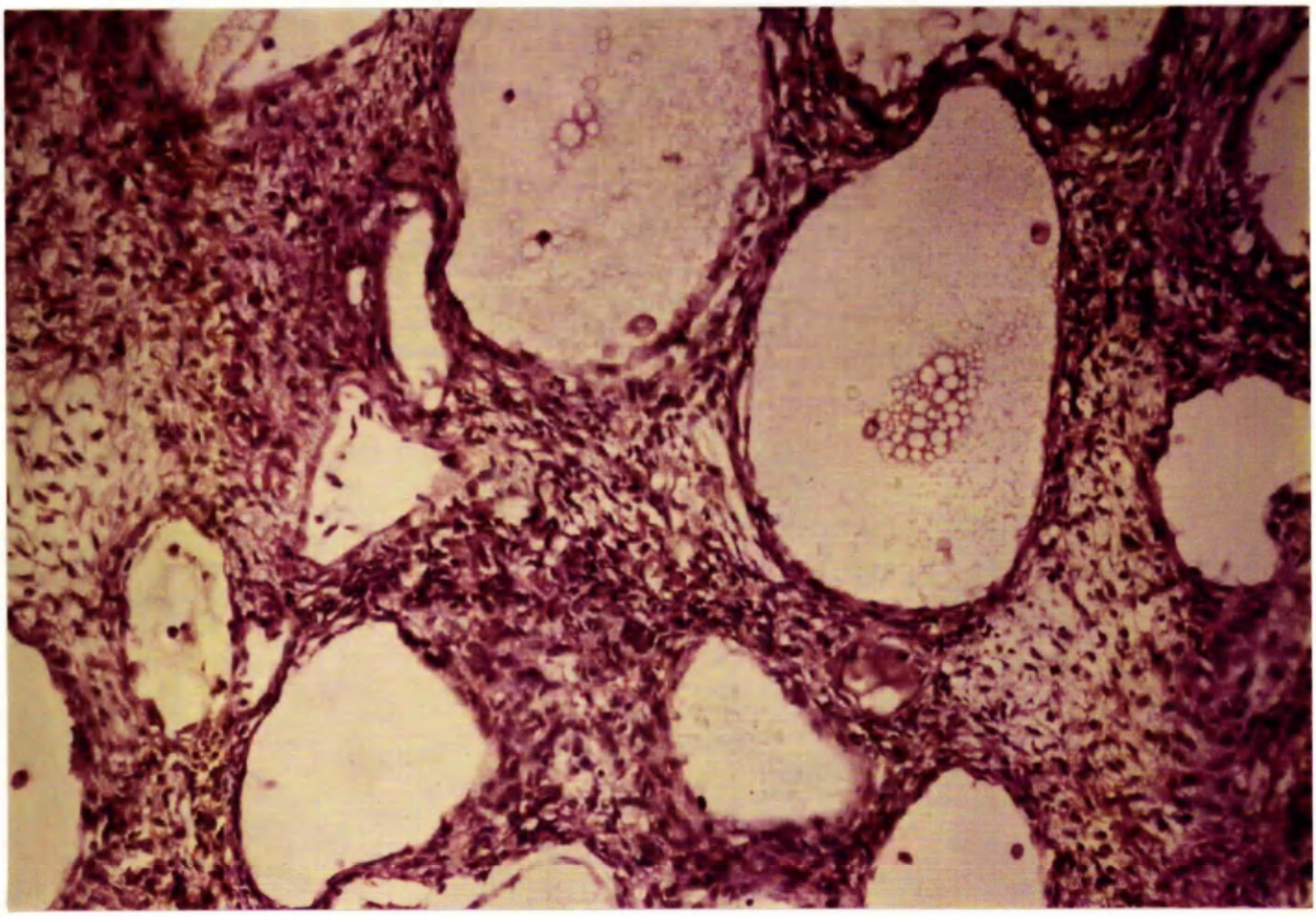


Fig. 6.4d.

Fig. 6.4. Histological patterns seen in yolk sac tumours.

Fig. 6.4e. Anaplastic YST. In some areas of YST, the tumour has a much more pleomorphic appearance with numerous mitotic figures. It consists of solid sheets of cells, but cytoplasmic vacuolation is a prominent feature and the nuclei are fairly regular and vesicular. This pattern is similar to some MTU's, but MTU is more irregular and the cytoplasm is less vacuolated. Anaplastic YST can only be diagnosed with certainty if it merges with areas of better differentiated YST. (H & E X 720)
Extragonadal germ cell tumour in male aged 21 yrs. Pure YST.

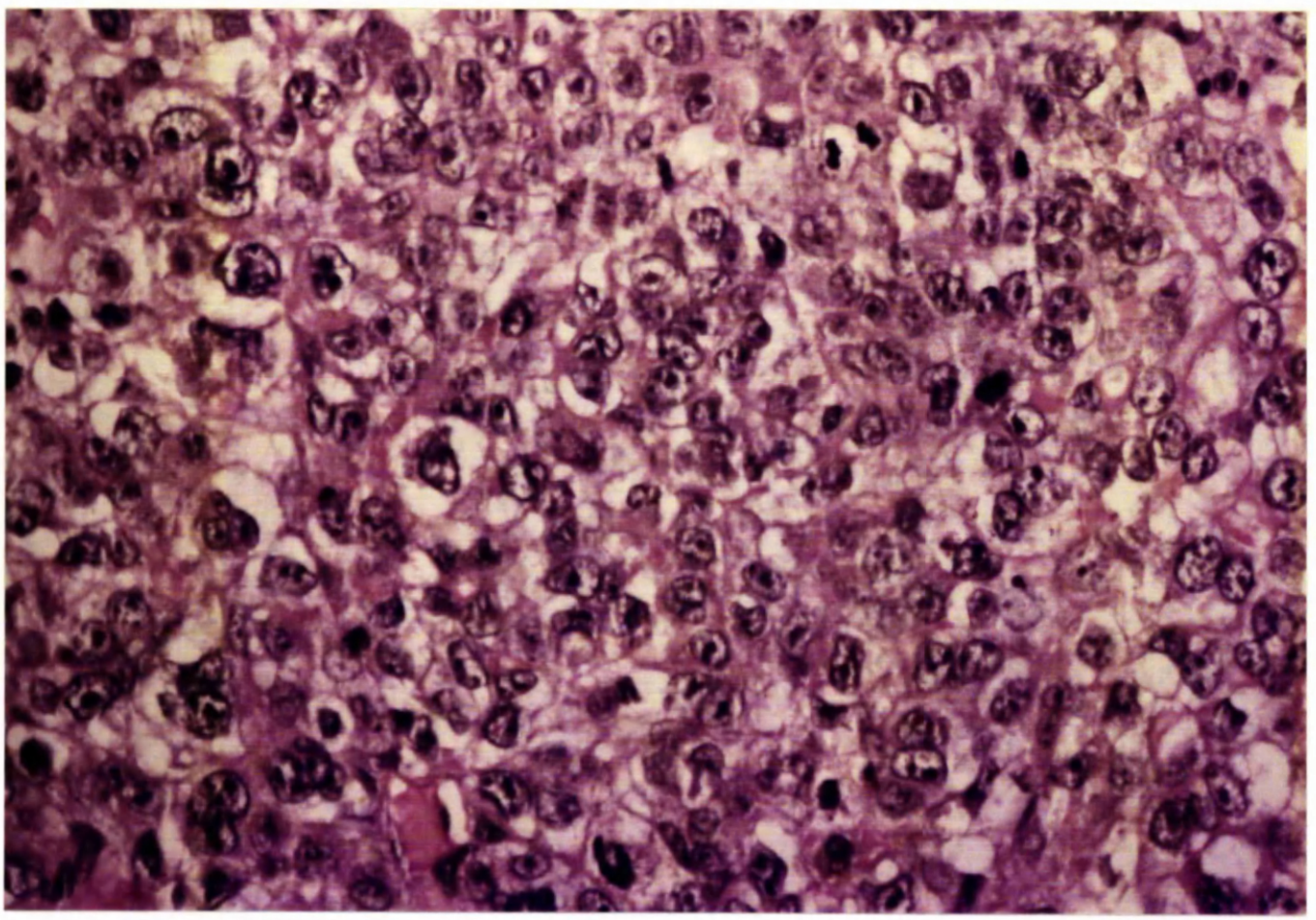


Fig. 6.4e.

Table 6. VIII. Incidence of yolk sac elements in association with other teratomatous elements.

Histology	Total number of teratomas	Number of teratomas with YST	Incidence
MTU	92	48	52 %
MTI	88	66	75 %
TD	13	5 *	38 %
extra embryonic	39	35	90 %
Total	232	153	66 %

* TD + YST would be classified as MTI in the British classification (Pugh, 1976).

4. Incidence of trophoblastic elements in teratomas

In the present group of 232 teratomas, 4 patients (2%) had pure MTT and a further 13 patients (6%) had teratomas containing MTT in association with other teratomatous elements. In addition, 80 teratomas (34%) contained isolated syncytiotrophoblasts which could be detected by immunoperoxidase staining for βHCG (Heyderman & Neville, 1976a). Therefore, 42% of teratomas contained trophoblastic elements and these were present in all age groups (Fig. 6.3).

5. Incidence of pure extra-embryonic teratomas

Of the 232 patients with teratoma, 39 contained only extra-embryonic elements without associated embryonic components, although 10 of these also contained seminomatous elements (Table 6.1X.)

6. Incidence of raised serum AFP in teratomas: association with YST

One hundred and sixty-eight (168) patients with teratomas had active disease at the time of serum sampling for AFP. Of these, 114 patients (68%) had evidence of YST and 119 patients (69%) had raised serum AFP at some stage in the course of the disease. However, as can be seen from Table 6.X., there is not an absolute and exclusive correlation between the two. In 53% of cases, YST was present and serum AFP was elevated, and in 16% of cases there was evidence of neither. However, 15% of patients had YST identified histologically although elevated serum AFP was never detected in the serum samples assayed, and 16% had raised serum AFP without histological evidence of yolk sac elements in the sections available for examination.

Table 6. IX. Details of germ cell tumours consisting entirely of extra-embryonic teratomas.

Age and site

infantile testis	3	(all pure yolk sac tumours)
adult testis	32	
adult extragonadal	4	
total	39	

Histopathology

YST	16
YST + trophoblasts	9
YST + trophoblasts + seminoma	4
YST + seminoma	6
MTT	4
total	39

Table 6.X. Incidence of yolk sac tumour and elevated serum AFP in 168 patients with active teratoma.

Yolk sac tumour *		Serum AFP	
Present	114 (68 %)	elevated	89 (53 %)
		normal	25 (15 %)
Absent	54 (32 %)	elevated	27 (16 %)
		normal	27 (16 %)
Total	168 (100 %)		168 (100 %)

* Alone or in combination with other germ cell elements

7. Serum AFP in follow-up studies and therapeutic monitoring

All 322 patients with testicular tumours and extragonadal germ cell tumours had serum AFP measured, and most had sequential measurement at each hospital visit thus enabling the course of the disease and the effect of therapy to be monitored serologically. A normal serum AFP could indicate one of the following possibilities:-

(a) The patient was tumour free

(b) Seminoma was present

(c) MTT was present

(d) The patient had active non-AFP-producing teratoma

(e) The patient had a small AFP-producing teratoma but insufficient to result in elevation of serum levels above the accepted upper level of normal (25ng/ml).

(f) AFP production had been suppressed by therapy although the tumour was still growing.

Therefore a negative serum AFP had little clinical significance, but a confirmed elevated serum AFP in the present series always indicated the presence of active teratoma. In some cases, the level of serum AFP was an accurate monitor of the extent of the disease in the body (Fig. 6.5).

A preoperative serum level is a very useful baseline measurement for the serological monitoring of the effect of surgery and subsequent therapy. Complete surgical eradication of tumour always resulted in serum AFP returning to normal, whereas failure to return to normal always indicated residual disease (Fig. 6.6). Unfortunately only 5 preoperative samples were available because of the referral system used, but the rate of fall of postoperative serum AFP could be used as an

Fig. 6.5. Serum AFP levels in an infant who had a left orchidectomy ($O^{\underline{x}}$) for a pure yolk sac tumour at the age of 3½ months. His AFP level was high postoperatively in spite of negative clinical examination, chest X-ray (CXR), lymphogram (LG) and intravenous pyelogram (IVP). It fell almost to normal and increased again to 40,000 ng/ml although LG remained negative. Examination under anaesthesia (EUA) revealed a left loin recurrence which largely regressed with chemotherapy using Vincristine (V), Actinomycin D (A), Cyclophosphamide (C) and Adriamycin (AD), and this was accompanied by a fall in serum AFP. At subsequent laparotomy, residual tumour in the left renal hilar lymph node, together with the adjacent adherent kidney, were removed. No other intra-abdominal metastases were detected. Serum AFP fell to normal after the laparotomy and further AD therapy, and he remains tumour-free and in good health.

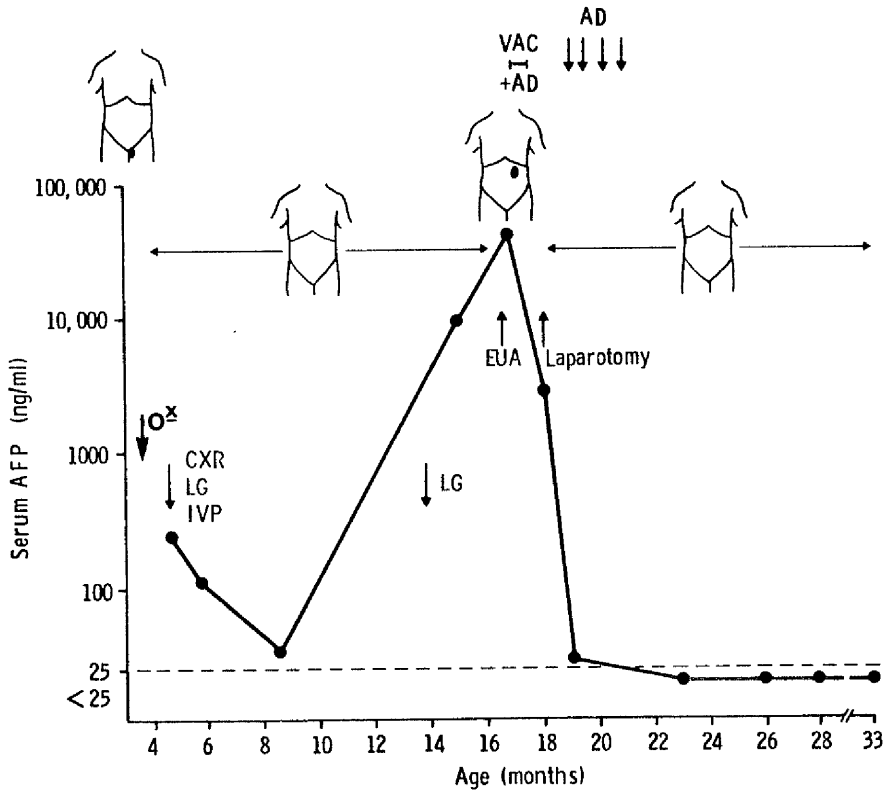

AD
VAC
+AD
100,000
10,000
1000
100
25
<25
Serum AFP (ng/ml)
Ox
CXR
LG
IVP
LG
EUA
Laparotomy
4
6
8
10
12
14
16
18
20
22
24
26
28
33
Age (months)

Fig. 6.5.

Fig. 6.6. Serum AFP in a male aged 31 years presenting with a right testicular tumour and left iliac nodes, left supra-clavicular node, lung and liver metastases. Chemotherapy with Vinblastine (V), Actinomycin D (A) and Methotrexate (M) caused tumour regression and a fall in serum AFP, but normal values were not reached even after orchidectomy ($O^{\underline{x}}$) which revealed a pure yolk sac tumour. Serum values rose to 3,300 ng/ml but following chemotherapy and radiotherapy (RT) with additional Actinomycin D (Act.D) he went into complete clinical remission including regression of para-aortic and right eye metastases. A subsequent rise in serum AFP preceded by several months clinical evidence of recurrent tumour when a liver scan showed hepatic involvement. Chemotherapy again resulted in a fall in AFP level to normal, but his metastatic disease progressed causing his death. Autopsy showed widespread undifferentiated teratoma with no evidence of yolk sac tumour.

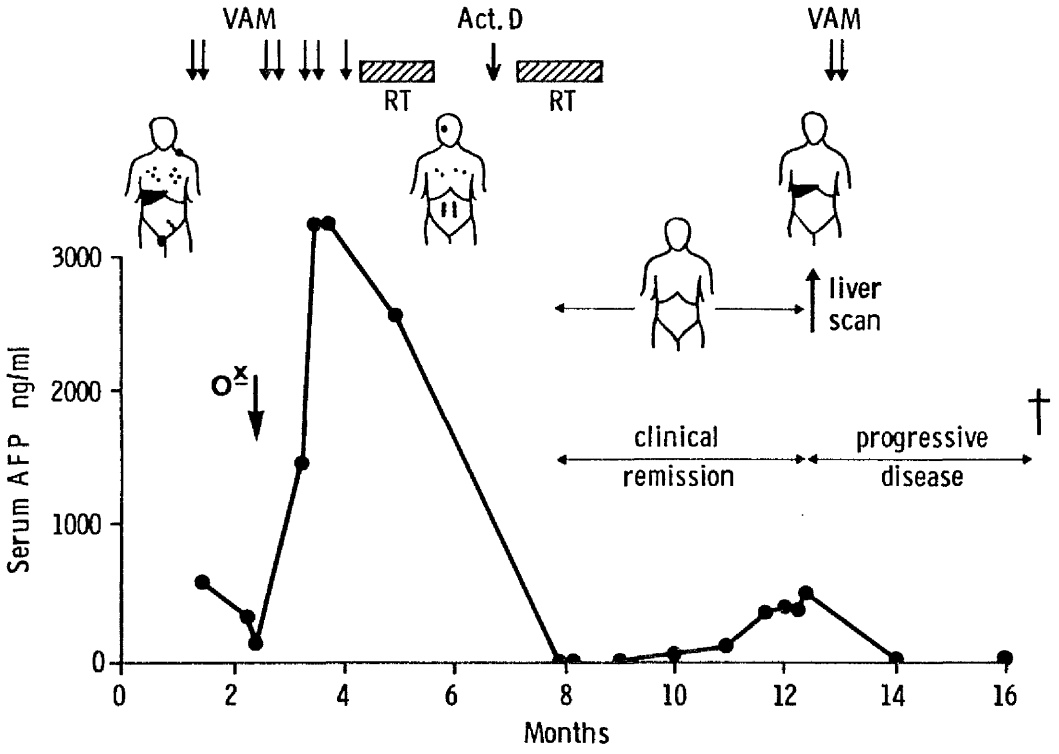

VAM
Act. D
VAM
RT
RT
liver
scan
clinical
remission
progressive
disease
†
Serum AFP ng/ml
3000
2000
1000
0
0
2
4
6
8
10
12
14
16
Months

Fig. 6.6.

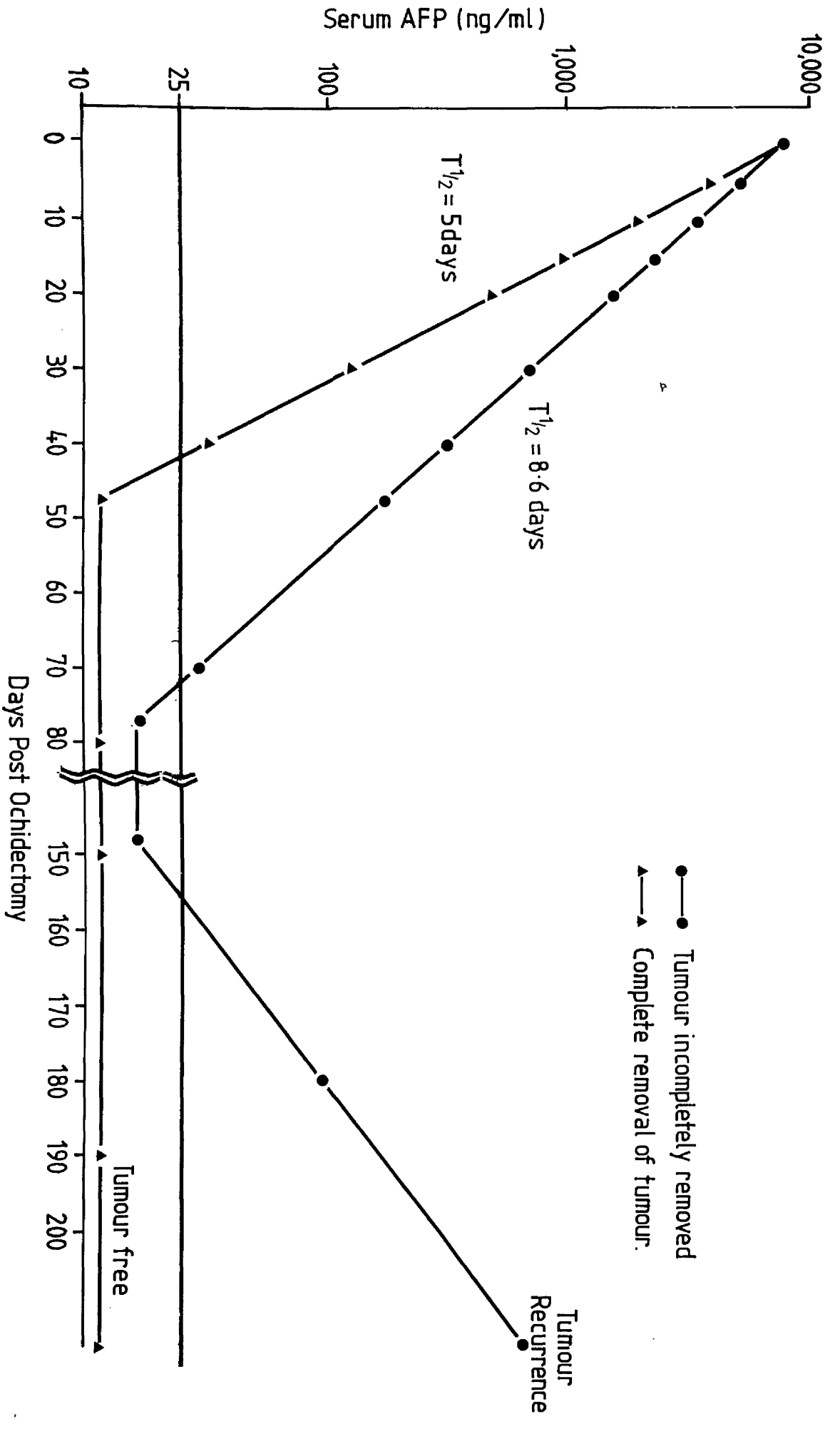
assessment of surgical completeness. In our laboratory (J. Kohn, personal communication) the half life of AFP in the serum was found to be 5 days, and if serum levels fell more slowly than expected then residual tumour was always present (Fig. 6.7). However, false negatives also occurred. In some cases, postoperative serum AFP fell to normal values with a half life of 5 days, but residual tumour was present and the tumour recurred at a later date.

Serum AFP fell to normal as a result of therapy (surgery, chemotherapy, radiotherapy) in 50 patients: in 31 of these, the tumour was completely eradicated, but in 19 cases incomplete regression occurred and the tumour recurred in spite of the period of normal serum values. In a further group of 19 patients under treatment, falling serum AFP coincided with clinical evidence of progression.

A secondary rise in serum AFP always indicated tumour recurrence: this was noted in 31 patients of whom 13 had raised serum AFP detected before the recurrence was evident by clinical and radiological examination (lead time: 1-30 weeks). In 14 patients clinical evidence of recurrence occurred before the serum AFP was elevated (lag time: 4-40 weeks), and in 4 patients clinical and serological evidence of recurrence occurred simultaneously. Six patients with AFP-producing tumours went into complete clinical remission as a result of therapy and serum AFP fell to normal, but tumour recurred at a later date without a secondary rise in AFP level.

It is clear that in many instances there is discordance between the changing levels of serum AFP and the progression or

Fig. 6.7. Graph of AFP levels in the serum of two hypothetical patients showing the significance of rate of fall of serum AFP postoperatively. The half life of AFP in the serum is 5 days. After complete excision of an AFP-producing tumour, serum levels fall exponentially with a half life of 5 days till normal baseline levels are reached (lower curve). A secondary rise does not occur. If the postoperative rate of fall of serum AFP has a greater half life than 5 days (e.g. 8.6 days: upper curve) then there is a residual source of AFP production in the body, i.e. incomplete removal of tumour. Serum levels may fall into the normal range, but increasing bulk of tumour will cause a secondary rise in serum AFP indicating tumour recurrence.


Serum AFP (ng/ml)
10,000
1,000
100
25
10
0
10
20
30
40
50
60
70
80
150
160
170
180
190
200
Days Post Ochidectomy
T½ = 5days
T½ = 8·6 days
Tumour incompletely removed
Complete removal of tumour.
Tumour Recurrence
Tumour free

regression of tumour in the body. However, in the majority of cases, rising or falling serum AFP positively reflected changes in tumour bulk. On 63 occasions tumour regression was accompanied by falling serum AFP, and in 37 cases, rising serum AFP coincided with tumour progression.

Four patients with raised serum AFP levels had an initial diagnosis of pure seminoma made on available histological material. Two of these patients had clinical evidence of active tumour at a time when a serum sample indicated elevated AFP levels: review of the histology of the primary tumour revealed teratomatous elements. The other 2 patients had normal serum AFP values initially, but raised levels developed later in the course of the disease: review of the histology of primary tumour confirmed that only seminomatous elements could be identified, but biopsies of recurrent tumours showed teratomatous elements (Fig. 6.8).

8. Prognostic features of teratomas

a) Effect of clinical stage

The significance of clinical stage at presentation on the mortality of 119 patients with teratoma followed prospectively for a minimum of 2½ years is shown in Table 6.XI. Sixty-five (55%) of these patients died of malignant disease, the vast majority of deaths occurring in patients with stage IV teratoma.

b) Effect of histological evidence of YST

The prognostic significance of YST in these 119 teratoma patients is shown in Table 6.XII. Pure YST carries a poor prognosis, worse than teratoma without yolk sac elements,

Fig. 6.8. Serum AFP levels in a male patient aged 31 years presenting with a testicular tumour diagnosed as a stage I pure seminoma by orchidectomy (O^{X}_{-}) and lymphangiography (LG). He had postoperative radiotherapy (RT). A second pure seminoma in the contralateral testis was removed 6 months after the initial operation. Within 3 months, chest X-ray (CXR) revealed lung hilar lymphadenopathy which was treated with Vinblastine (V), Actinomycin D (A) and Methotrexate (M), but he developed inferior vena caval compression (IVC comp) with lower limb oedema. Serum AFP was first measured at this stage and was found to be in the normal range. In spite of radiotherapy, the mediastinal tumour mass increased in size and serum AFP increased to very high levels (6,600 ng/ml). Chemotherapy with Cyclophosphamide (Cyclo), Adriamycin (Adria) and Dimethyl triazeno imidazole carboxamide (DIC) did not prevent tumour growth, and encroachment on the oesophagus caused dysphagia. A preterminal thoracotomy revealed an inoperable tumour which was biopsied and found to be teratomatous (MTI + YST).

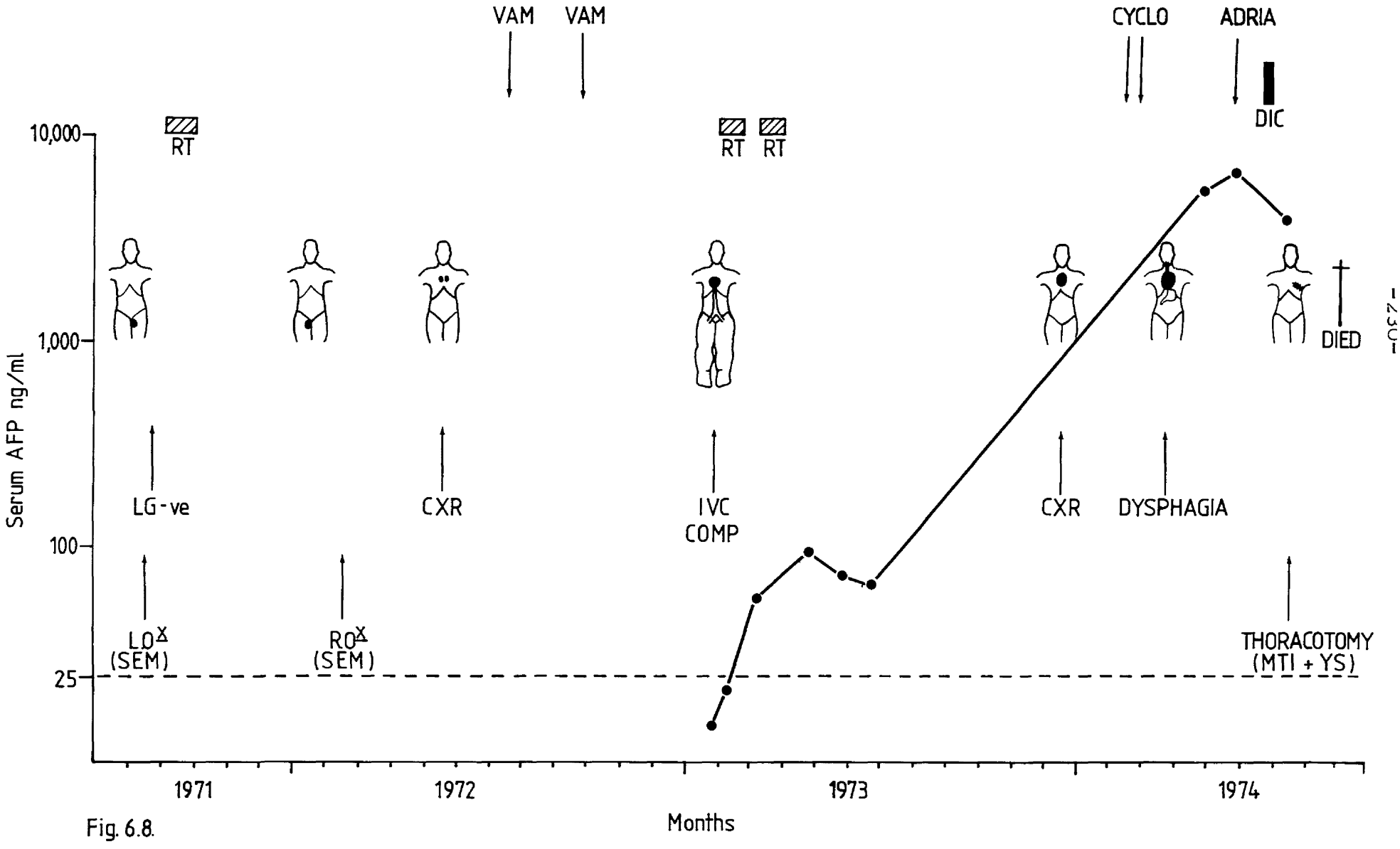

VAM
VAM
CYCLO
ADRIA
DIC
RT
RT
RT
10,000
1,000
100
25
Serum AFP ng/ml
LG-ve
CXR
IVC
COMP
CXR
DYSPHAGIA
DIED
LO^X
(SEM)
RO^X
(SEM)
THORACOTOMY
(MTI + YS)
1971
1972
1973
1974
Months

Fig. 6.8.

Table 6.XI. Correlation between clinical stage at presentation and mortality in 119 teratoma patients followed for at least $2\frac{1}{2}$ years.

Clinical stage at presentation	Number of patients		
	dead	alive	total
I	1	25	26
II	0	12	12
III	1	4	5
IV	63	13	76
Total	65	54	119

Table 6.XII. Correlation between histological evidence of YST and mortality in 119 patients with teratoma followed for at least $2\frac{1}{2}$ years.

Histology	Dead	Alive	Total
pure YST	14 (70 %)	6 (30 %)	20
YST + other teratomatous elements	28 (53 %)	25 (47 %)	53
teratomas with no evidence of YST	23 (50 %)	23 (50 %)	46
Total	65 (55 %)	54 (45 %)	119

and worse than YST combined with other teratomatous elements. Table 6.XIII indicates that the presence of YST in MTU has little effect on prognosis of MTU, but yolk sac elements adversely effect the prognosis of MTI. However, MTI + YST has a better prognosis than MTU with or without YST.

c) Effect of raised serum AFP

In this group of 119 patients followed for at least 2½ years, 85 had serum AFP measured at a time when the tumour was active. The survival of these patients and the prognostic significance of raised serum AFP is shown in Table 6.XIV. Evidence of AFP production has a marginally adverse effect on the prognosis of teratoma taken as a whole. Although numbers are small, a raised serum AFP associated with MTU or MTI is probably an adverse sign (Table 6.XV).

9. Other tumour index substances

Human chorionic gonadotrophin (HCG) was measured in the urine or serum of some patients with testicular and germ cell tumours. Both AFP and HCG were measured in 102 patients with active teratoma and 18 patients with active seminoma (Table 6.XVI). Both markers were elevated in 54% of the teratoma patients, and only 6% of teratoma patients had neither elevated. Seminomas were not associated with raised serum AFP, but 44% of patients with active seminoma had raised HCG levels in the urine or serum.

Table 6.XIII. Relative significance of YST, MTI and MTU with respect to mortality in 112 patients with teratoma followed for at least $2\frac{1}{2}$ years.

Histology	Dead	Alive	Total
MTU	16 (64 %)	9 (36 %)	25
MTU + YST	15 (65 %)	8 (35 %)	23
MTI	3 (21 %)	11 (79 %)	14
MTI + YST	13 (43 %)	17 (57 %)	30
pure YST	14 (70 %)	6 (30 %)	20
Total	61	51	112

Table 6.XIV. Correlation between serological evidence of AFP production and mortality in 85 teratoma patients followed for at least $2\frac{1}{2}$ years.

Serum AFP	Number of patients		
	Dead	Alive	Total
elevated *	45 (78 %)	13 (22 %)	58
normal **	19 (70 %)	8 (30 %)	27
Total	64 (75 %)	21 (25 %)	85

* Serum AFP greater than 25 ng/ml at some time in the course of the disease

** Serum AFP never greater than 25 ng/ml although disease was active at some stage in the course of serum sampling

Table 6.XV. Significance of AFP production with respect to mortality in 80 patients with active MTI, MTU or YST followed for at least $2\frac{1}{2}$ years.

Histology	Serum AFP	Number of patients		
		Dead	Alive	Total
MTU *	elevated	18 (82 %)	4 (18 %)	22
	normal	13 (68 %)	6 (32 %)	19
MTI *	elevated	14 (70 %)	6 (30 %)	20
	normal	1 (33 %)	2 (67 %)	3
YST **	elevated	12 (86 %)	2 (14 %)	14
	normal	2 (100 %)	0	2
Total		60	20	80

* Some of these teratomas contained extra-embryonic elements in addition to MTI or MTU

** Pure extra-embryonic teratomas

Table 6.XVI. Tumour markers in 120 patients with active germ cell tumour at a time when both AFP and HCG were measured.

Tumour markers in serum (or urine)	Number of patients	
	Active teratoma (± seminoma)	Active seminoma
Neither AFP nor HCG elevated	6 (6 %)	10 (56 %)
AFP only elevated *	19 (19 %)	0
HCG only elevated **	22 (22 %)	8 (44 %)
Both AFP and HCG elevated	55 (54 %)	0
Total	102	18

* Serum AFP greater than 25 ng/ml

** Serum βHCG greater than 2 ng/ml by RIA or Urinary HCG greater than 100 iU per 24 hours by pregnancy test

C. DISCUSSION

There are many authoritative classifications of testicular tumours (Mostofi & Price, 1973; Mostofi & Sobin, 1977; Pugh, 1976; Teilum, 1976). The classification of the British Testicular Tumour Panel and Registry (Pugh, 1976) is straightforward for the histopathologist, readily understood by the clinician (Peckham & McElwain, 1975) and correlates well with the clinical behaviour of the tumour (Bar & Hedinger, 1976). However, it is mainly a prognostic classification rather than a histogenic or functional classification and is therefore limited in its ability to indicate the possible tumour markers which may be elevated in the serum. This deficiency can be partially overcome by incorporating the criteria for diagnosing extra-embryonic components as proposed by Teilum (1976), and by identifying isolated syncytiotrophoblasts by immunocytochemical staining methods.

In the present series of 316 germ cell tumours, 56% were teratomas (non-seminomatous germ cell tumours), 27% seminomas and 18% combined tumours. In the teratoma ($\pm$ seminoma) group 74% had metastatic disease at the time of referral to the Royal Marsden Hospital, and histologically 3.4% had TD. Comparing these figures with the results of Pugh (1976), the present series contains a low porportion of seminomas and TD, and a high proportion of tumours of high grade malignancy. Also, the present series contains a higher proportion of tumours which have metastasised beyond the testis. This suggests that there was a certain degree of patient selection in the present series, tumours with a poor prognosis being more prevalent, a feature

which must be borne in mind when the significance of the results is discussed.

The results may also be biased because of sampling error. As a result of the referral system used, preoperative serum samples were seldom available, and the limited number of histological sections available may not have been fully representative of the complete range of structures present in any one tumour. In addition, any temporal changes in morphological patterns could not be monitored because tumours could only be sampled at a single instance in time.

1. The use of serum AFP as a tumour index substance

Serum AFP is elevated in association with many neoplastic and non-neoplastic conditions (see introduction) and is therefore not a specific marker for testicular tumours. However, in the present series, a confirmed raised serum AFP in an adult always indicated the presence of active teratoma and could rule out pure seminoma, pure trophoblastic tumour, and non-germ cell testicular tumour. Successful therapy always resulted in a fall of serum AFP levels with a half-life in the serum of 5 days; and a subsequent rise always indicated recurrent disease. If the rate of fall of serum AFP after surgery had a greater half-life than 5 days, then residual tumour was present. The lack of false positives in this series suggests that serial measurements of serum AFP is a very good adjunct to clinical examination in the follow-up of patients with germ cell tumour. However, its usefulness is limited by the large number of false negatives which may occur.

One third of teratomas are not associated with raised serum AFP, and of those that do produce AFP, serum levels give no absolute indication of tumour bulk, and often fluctuations of tumour size and activity are not reflected in the level of AFP in the serum (Fig. 6.6). However, in the cases where serum AFP accurately reflects the extent of the tumour in the body (Fig. 6.5), its serial determination is a valuable aid to the monitoring of the disease . Serum AFP often detects recurrent disease before it is clinically apparent (Figs. 6.5 & 6.6), but in other patients, clinical and radiological methods are more sensitive. Therefore serum AFP is not a substitute for meticulous clinical examination on the follow-up period.

2. Other tumour index substances

The measurement of more than one serum marker increases the usefulness of serological monitoring of teratomas. In the present series, 94% of teratoma patients had elevation of at least one of serum AFP, serum βHCG, or urinary HCG. The levels of these markers may fluctuate discordantly with therapy (Braunstein et al., 1976), therefore serial estimation of both is desirable.

In a recent review of secretory products released by germ cell tumours (Grigor et al., in press) the importance of serial serum measurements of both AFP and βHCG in patients with testicular tumours was stressed. These two substances are the most specific and the most consistently elevated germ cell tumour markers, and they give the most accurate assessment of tumour progression and regression. Other tumour markers may be elevated in the serum, but none has yet been found to be better than AFP and βHCG.

Carcinoembryonic antigen (CEA) may show slight transient elevations in the serum of teratoma patients, but these levels are unrelated to AFP levels and do not give an accurate indication of tumour bulk nor tumour progression (Wahren & Edsmyr, 1974). Talerman et al., (1977) state that serum measurement of CEA is of no value in the monitoring of germ cell tumours; however, elevated CEA levels may indicate differentiation towards gastrointestinal structures within teratomas.

An iron-containing oncofetal antigen called α_2-H globulin has been detected in the serum of some patients with malignant disease (Buffe et al., 1968). This globulin was later found to be ferritin, and is elevated in the serum of some patients with teratomas (Laurence & Neville, 1972). Pedersen et al. (1976) confirmed that some germ cell tumours are associated with raised serum ferritin levels, but these levels are not related to AFP levels, and do not reflect tumour activity.

Human placental lactogen (HPL), also known as human chorionic somatomammotrophin (HCS), is another placental hormone produced by the syncytiotrophoblasts and is elevated in maternal serum during pregnancy. Some germ cell tumours, especially if chorionic elements are present, are associated with raised serum levels, and it is postulated that HPL in conjunction with elevated oestrogen levels are responsible for gynaecomastia in patients with testicular tumours (Frantz et al., 1965; Greenwood et al., 1971). However, HPL is less consistently produced by chorionic elements than βHCG (Frantz et al., 1965; Stepanas et al., 1978), and as with βHCG, HPL is not specific for trophoblastic tissues (Weintraub & Rosen, 1971).

Bohn (1972) described a pregnancy specific β_1-glycoprotein ($PS\beta_1G$) which Horne et al. (1976,1977) have demonstrated in the syncytiotrophoblasts of normal placentae, and also in gestational choriocarcinomas and trophoblastic elements in malignant testicular teratomas. Searle et al. (1978) have shown that, in addition to choriocarcinomas and teratomas, this glycoprotein may be elevated in the serum of patients with tumour of the breast, colon and ovary, as well as in the serum of some healthy males and non-pregnant females. Therefore, $PS\beta_1G$ is not a specific marker for trophoblastic tumours. In choriocarcinomas, βHCG levels in the serum are greater than $PS\beta_1G$ levels, and βHCG is a more useful tumour marker in the monitoring of the tumour. However, in some cases of malignant teratoma, $PS\beta_1G$ levels may excede βHCG levels, and may be more useful in the detection of residual minimal disease (Searle et al.,1978).

Many patients with malignant disease have elevated serum acute phase reactive proteins which are produced by the liver in times of stress or injury (Koj, 1975). These serum markers are not specific for tumours, but may give an indication of the extent of tumour spread. $Alpha_1$-antitrypsin (AAT) is an acute phase reactive protein which is elevated in the serum of some patients with testicular tumours, and which has been demonstrated within teratoma cells (Palmer et al., 1976). However, this does not necessarily indicate production by tumour cells, and AFP is a more specific and more sensitive marker.

Kadish et al. (1976) described a case of primary retroperitoneal germinoma (seminoma) with elevated serum levels of lactic dehydrogenase (LDH). Radiotherapy resulted in complete

tumour regression, and serum LDH levels returned to normal. This suggests that in some cases, serum LDH levels may accurately monitor the course of a germ cell tumour, but LDH is not a specific marker for tumour activity.

Patients with testicular tumours often have gynaecomastia, and this is considered to be a poor prognostic sign, especially if galactorrhoea is also evident. Stepanas et al. (1978) studied 45 patients with testicular tumours, 27 of whom had gynaecomastia. Serum levels of 6 hormones were measured, viz. prolactin (PRL), human placental lactogen (HPL), βHCG, testosterone, oestrone (E_1) and oestradiol(E_2). Levels of testosterone tended to be low, but the other hormones were often present in the serum in elevated amounts. Tumour HCG was thought to stimulate aromatisation of steroid precursors within tumour cells with resultant oestrogen production. The oestrogens subsequently stimulated pituitary secretion of prolactin. Gynaecomastia formation in these patients tended to be suppressed by high circulating testosterone levels. Stepanas et al. (1978) suggested that these hormones, especially oestrone, should be measured in the serum of all patients with testicular tumours as tumour markers in the monitoring of the course of the disease.

It is evident that testicular tumours may be associated with abnormal levels of many substances in the serum. The complete assessment of patients with testicular tumours should involve serial serum monitoring of all substances which may be affected, but such a comprehensive follow-up protocol is impractical for routine monitoring of all testicular tumour patients. Frequent thorough clinical examination is mandatory; and accurate

clinical staging using all available technology (Table 6.II) is the most important index of prognosis. Serological monitoring is a very important and useful adjunct to clinical examination and should be considered as a necessary part of routine follow-up. At present, serum AFP and βHCG levels are the most useful known markers for monitoring the progress of testicular tumours, and both should be measured at frequent intervals. However, in a few patients, other markers may have more to offer, and these should be considered in cases where AFP and βHCG levels are found to be unrepresentative of the activity and extent of the tumour.

3. Correlation between serum AFP levels and tumour morphology

There is a strong correlatiion between raised serum AFP and the presence of yolk sac elements in teratomas, and some authors claim that AFP is a specific marker for YST (see introduction). However, the results presented in this thesis indicate that there is not an absolute and exclusive correlation between the two, 15% of patients having raised serum AFP without YST being recognised in the available histological sections, and 16% of patients having yolk sac elements but serum AFP within the normal range (<25ng/ml). This discrepancy may be explained by sampling error. It is possible that patients with raised serum AFP levels but no histological evidence of YST had yolk sac elements present in parts of the tumour which were not examined histologically. Also, it is possible that patients with YST identified in their tumour but no evidence of raised serum AFP levels had raised levels at a time when the serum was not sampled: and when their serum was tested they may have had

active teratoma without residual yolk sac elements. However, it is more likely that some yolk sac tumours do not produce AFP, and that elements other than YST are capable of its production. The discrepancy between AFP production and the presence of YST is also evident when considering the prognosis of testicular tumours (vide infra).

AFP in teratomas had been demonstrated in yolk sac structures by immunocytochemical staining (see introduction), but this technique is not always reliable for AFP localisation (Heyderman & Neville, 1976b). AFP is soluble in formol saline fixative (Grigor & Kohn, unpublished observations) therefore diffusion may have occurred in formalin fixed tissue. Also formaldehyde inhibits the binding of anti-AFP antibodies to AFP, therefore routine formaldehyde fixed paraffin sections must be washed meticulously in order to remove all traces of fixative.

AFP is produced in the embryo and foetus by the yolk sac, liver and gastrointestinal tract, therefore hepatoblastic or gastrointestinal differentiation in teratomas may account for AFP production. Engelhardt et al. (1973) demonstrated AFP in mouse teratomas in regions showing differentiation towards endodermally derived endothelium, and Kurman et al. (1977) have shown that large mononuclear cells in embryonal carcinomas (MTU) contain AFP. Abelev (1967) suggested that AFP production in teratomas may be due to hepatoblastic cell, and the cells described by Kurman et al. (1977) are possibly hepatoblasts. The anterior mediastinum is a common site for extragonadal germ cell tumours (Luna et al., 1976). Wepsic et al. (1976) described an anterior mediastinal "anaplastic carcinoma with hepatoblastic" appearance

associated with high serum AFP: this tumour may have been a teratoma with differentiation towards hepatoblasts thus accounting for the AFP production.

A female patient with an ovarian teratoma containing neural elements had a raised serum AFP (Esterhay et al., 1973). If the foetal neural tube is exposed to the amniotic fluid in utero due to neural tube defects, amniotic fluid AFP is higher than normal indicating that perhaps neural tissue produces AFP. Breborowitz & Mackiewicz (1977) demonstrated that embryonic brain in tissue culture produces AFP. Therefore, neural elements within a teratoma may account for AFP production.

Since a variety of elements in teratomas are theoretically capable of producing AFP, it is difficult to argue that AFP is a specific marker for yolk sac elements. Parkinson & Beilby (1977) state that many cases of embryonal carcinoma or MTU of the testis should be reclassified as YST because of AFP production, but such a conclusion is unwarranted because of lack of specificity of AFP production.

4. Incidence of yolk sac elements in teratomas

Very few published figures are available which indicate the incidence of YST in teratomas because there is confusion and disagreement about the diagnostic criteria for its recognition, and because very few standard text books describe fully the histological features. Teilum (1976) has recently described the morphological features which he accepts as YST differentiation, and these criteria have been accepted by many authors and by the WHO classification of testicular tumours (Mostofi & Sobin, 1977). However, Pugh (1976) denies the existance of YST in germ cell tumours of adults, and Parkinson & Beilby (1977) at the opposite extreme suggest that all non-organoid

(non-somatic) structures in non-seminomatous germ cell tumours should be regarded as extraembryonic elements.

Teilum (1976) studied a selected group of teratomas with a definite bias towards those with yolk sac elements and therefore his series does not indicate the true overall incidence of YST in teratomas.

Pugh (1976) states that the incidence of YST in germ cell tumours of adults is nil. Parkinson & Beilby (1977) claim that 89% of 92 teratomas contained YST but this figure must be regarded with caution because of their broad acceptance of yolk sac differentiation. Talerman (1975) used Teilum's classification and found YST in 38% of 68 teratomas of adult testes, but stated that pure YST does not occur in adult testicular tumours. Wurster et al. (1972) found YST in 37% of 122 teratomas of adult testes.

In the present series there was probably some degree of patient selection towards those with more aggressive tumours. In our group, 66% of 232 teratomas in males contained yolk sac elements, and YST was found in all age groups: the porportion of teratomas containing YST remained fairly constant in all the age groups. Pure YST was found in adults as well as in tumours of the infantile testes.

The exact incidence of YST will not be known until the criteria for its diagnosis is standardised and examined in many large series of unselected teratomas.

5. Features of prognostic significance in teratomas

The most important prognostic indicator of the outcome of testicular tumours is the clinical staging (Table 6.XI).

The best histological classification of germ cell tumours giving an indication of prognosis is that of Pugh (1976) as discussed by Bar & Hedinger (1976). Seminomas and TD's have a good prognosis whereas MTT is usually rapidly fatal and is the most sinister histological feature. The prognosis of MT1 and MTU lie between these two extremes, MT1 having a better prognosis than MTU (Pugh, 1976). The present series vindicates these conclusions. Parkinson & Beilby (1977) find no difference in prognosis between MT1 ("Mixed germ cell tumours") and MTU ("Yolk sac tumours") but our series agrees with Pugh (1976) that MT1 carries a better prognosis than MTU.

Many authors claim that the presence of YST (Parkinson & Beilby, 1977; Roth & Panganiban, 1976; Sell et al., 1976; Talerman, 1975) or a raised serum AFP (Abelev, 1971; Ballas, 1974; Bourgeaux, et al., 1976; Norgaard-Pederson et al., 1975) adversely affect the prognosis of germ cell tumours. In the present series, the presence of YST in MT1 had an adverse prognostic effect, but MT1 + YST had a better prognosis than MTU, and the presence of YST had little or no effect on the prognosis of MTU (Table 6. XIII). Pure YST in adults carries a poor prognosis. Evidence of AFP production probably has a marginally adverse effect on the prognosis of teratomas (Tables 6.XIV & 6.XV) but numbers are still too few to make a definite conclusion. These results indicate a further discrepancy between the presence of YST and AFP production. A raised serum AFP adversely affects the prognosis of MTU (Table 6.XV) whereas the prognosis of MTU is unaffected by the presence of YST (Table 6.XIII).

SECTION II : HUMAN TUMOURS

CHAPTER 7

ADRENOCORTICAL TUMOURS

CHAPTER 7

ADRENOCORTICAL TUMOURS

This chapter is a continuation of the general theme of the thesis: the correlation of morphology and function in tissues, using steroid-producing glands and human neoplasms as examples. The normal foetal testis is a steroid-secreting organ whose functions were described and discussed in Section I. In the first part of Section II, I gave an account of germ cell tumours of the testis in man, and the association between morphological patterns and secretory products was considered. However, in such tumours, steroid production is not a major feature and therefore steroidogenesis was not discussed in depth.

Adrenocortical tumours, like foetal testes, have the capacity to synthesise and secrete steroid hormones and/or precursors. Testicular germ cell tumours are usually malignant neoplasms, but in some cases the actual degree of malignancy cannot be assessed on histological grounds alone. Although the malignant potential of adrenocortical tumours may also be difficult to determine histologically, those studied by me were all clinically malignant. In this chapter I attempt to correlate the morphology of these adrenocortical tumours with their steroidogenic capacity and their degree of malignancy.

CHAPTER 7

NON-HORMONAL ADRENOCORTICAL CARCINOMAS

A. MATERIALS AND METHODS

1. Patients

Thirteen cases of "non-hormonal" adrenocortical tumours were seen at the Royal Marsden Hospital in London from 1948 to 1972. One case was excluded because the histological material was not available. The cases were referred primarily to the hospital for treatment rather than diagnosis. Some of the clinical and all of the histological data are available in a further 8 cases referred for pathological diagnosis to the University Department of Pathology, Glasgow Royal Infirmary, from 1960 to 1970. Their inclusion brings the total number of new cases to 20.

Histological examination was made on paraffin-embedded material sectioned at 6μ and stained with haematoxylin and eosin. Some specimens were received as paraffin-embedded blocks or previously stained sections. Surgical material was fixed in 10 per cent neutral formalin and trimmed prior to embedding.

The present series (Table 7.I) consists of 11 males and 9 females ranging in age from 2½ to 66 years; 10 of the patients were between 50 and 70 years of age. The right adrenal gland was affected in 9 patients and the left in 10. In one (Case 13), the site was not stated.

2. Signs and Symptoms

The commonest presenting features were a palpable mass (11/20) and pain (11/20)(Table 7.I). Anorexia was present

Table 7.I. Clinical details of 20 patients with non-hormonal adrenocortical carcinoma.

Case	Sex	Age	Signs, symptoms and radiographic studies
1	M	2½	5 days history of malaise, drowsiness, backache, right facial weakness and fever. IVP : ill-defined mass in right kidney. Metastases to right 8th and 10th ribs.
2	M	38	Weakness and tiredness for 5 years. 5 months prior to admission, heart failure and hepatomegaly. Pain right upper quadrant, palpable mass, weight loss, ankle oedema. Liver palpable 6 cm. IVP : ptosis of right kidney. Arteriogram : vascular component identified.
3	F	35	8 years left loin pain. 2 years hepatosplenomegaly of unknown aetiology, mass in left loin, shortness of breath. IVP : tumour of left adrenal gland.
4	M	38	Swelling of abdomen for 2 years. Pyrosis for 2 years. Mass right upper quadrant. IVP : large right renal mass. Mass above and larger than the kidney.
5	F	61	abdominal discomfort and increasing girth for 1 month.
6	F	56	Dragging sensation in epigastrium for 1 month, mass palpable, increasing nausea and vomiting, anaemia, weight loss and pain. Barium meal : carcinoma of stomach.
7	M	64	Pain right side and back. Haematuria. IVP : enlarged right kidney.
8	F	53	5 weeks pain in left flank, increasing girth, palpable mass, anorexia. IVP : downward displacement of left kidney. Pre-sacral air insufflation : adrenal mass. Aortagram : tumour vessels seen originating from aorta.
9	F	48	Backache, abdominal discomfort, anorexia, palpable mass for 1 month. IVP : mass left kidney
10	F	42	July '69 admitted with pain in left hypochondrium radiating to the left iliac fossa, shortness of breath. Neurofibromatosis. October '71 tiredness, anorexia, loin pain, nausea and vomiting, pain over 9th and 10th ribs. IVP : renal pelves enlarged, mass in left upper quadrant. Barium meal : mass in left hypochondrium. Spine : loss of pedicle of T 9. Pelvis : right acetabular deposit.
11	F	65	1966 right adrenal tumour removed. 1972 nausea, vomiting and anorexia. Mass in right loin and a skin nodule found. IVP : negative. Chest : multiple metastases in the lung.
12	M	66	Indigestion "for many years", 2 week history of lower abdominal pain. 2 stone weight loss and palpable mass in right upper quadrant. Barium meal : large liver and displacement of stomach to left. IVP : right kidney displaced downwards. Lymphogram : deposits in left external iliac and left para-aortic nodes.
13	M	-	Urinary obstruction and paraplegia due to dorsal vertebral metastases. Radiography : air insufflation revealed adrenal tumour.
14	M	59	Abdominal swelling in left upper quadrant for 1 year. Abdominal wall recurrence 3½ years postoperatively.
15	M	39	1 year "indigestion" and upper abdominal pain. Chest X - Ray : lung metastases 2 years postoperatively.
16	F	45	Chest X - Ray : lung metastases seen March '66. Mild abdominal pain and backache. IVP : left kidney displaced downwards. Spleen displaced upwards.
17	F	57	Asymptomatic. Chest X - Ray showed lung metastases.
18	M	50	Abdominal swelling, proptosis 3 years postoperatively. Skull X - Ray showed retro-orbital tumour confirmed by tomography and arteriography. Chest X - Ray showed lung metastases.
19	M	14	Chest pain, mass in right loin. Chest X - Ray : lung metastases. Arteriogram : blood supply of tumour from aorta.
20	M	61	3 stone weight loss in 1 year. Left abdominal pain. Chest X - Ray normal. IVP : left kidney displaced downwards. Arteriogram : adrenal tumour confirmed.

in six patients, while fever was recorded only once (a child aged 2½ years). In each of four patients, backache and vague dyspepsia were also observed.

3. Radiological studies

These investigations were carried out in most but not all patients (Table 7.I). The intravenous pyelogram was abnormal in 12/13 cases and revealed either a renal deformity, a supra-renal mass, or displacement of the kidney. The barium meal was abnormal in three patients and was interpreted as a primary gastric tumour in two cases (Cases 6 and 10). Presacral air insufflation and arteriography contributed to the diagnosis in six cases (Cases 6,8,10,13,19 and 20). A postoperative lymphangiogram (Case 12) revealed evidence suggesting left external iliac and para-aortic node involvement.

4. Steroid investigations

The principal investigations which were carried out are shown in Table 7.II. In a few instances, minimal aberration of cortisol metabolism may have existed but without causing overt clinical manifestations. In addition, increased excretion of the metabolites of pregnenolone, including pregn-5-ene-3α,16α, 20α-triol were present in the urine of two patients (Cases 3 and 20).

5. Treatment

Complete excision of the tumour was achieved surgically, in 11 patients (Cases 1,5,10-12,14-16,18-20,). The tumours of 3 (Cases 2,4 and 8) could only be partially removed, and in a

Table 7. II. Therapeutic and laboratory aspects of 13 patients with non-hormonal adrenocortical carcinoma.

Case	Postoperative radiotherapy*	Postoperative chemotherapy*	Laboratory tests
1	None	Methotrexate 10 mg/24 hrs	None relevant
2	2700 r in 35 days to tumour bed	o,p'-DDD 8 g/day for 2 months. Prednisone 15 mg/day	Urinary 17-oxogenic steroids† 135 mg/24 hrs; 66 mg/24 hrs Urinary 17-hydroxysteroids† 152 mg/24 hrs; 83 mg/24 hrs (after o,p'-DDD)
3	None	None	Blood and urine steroids reported slightly abnormal
4	None	None	Urinary 17-oxosteroids : 4.3 mg/24 hrs†
5	2740 r in 35 days	None	Urinary 17-oxosteroids : 7.5 mg/24 hrs†
6	Pituitary gold rods	None	None relevant
7	2340 r in 35 days	None	None relevant
8	None	None	Urinary 17-oxosteroids : 40 mg/24 hrs
9	2050 r in 21 days	None	Plasma cortisol : (0900) 24 µg/100ml (2400) 22 µg/100ml Urinary 17-oxosteroids : 23 mg/24 hrs Urinary 17-hydroxysteroids : 12 mg/24 hrs
10	Bone metastases. 3900 r in 21 days to T 9 3500 r in 30 days to femur	None	Plasma cortisol : 33.5 µg/100 ml VMA : normal Urinary 17-oxosteroids : 5 mg/24 hrs† Urinary 17-hydroxysteroids : 5 mg/24 hrs†
11	4896 r in 52 days to the tumour with minimal change in its size	2nd June '71 to 11th Dec. '71 : cyclophosphamide and vincristine sulphate. 3rd March '72 to 26th May '72 : methotrexate. 3rd June '72 to 4th June '72 : 5-fluorouracil (2 doses only) and o,p'-DDD 8 g/day. July '72 onwards : dexamethasone 2 mg/day.	Plasma cortisol : 3rd June '71† 13th June '72† 0900 47 µg/100ml 15 µg/100ml 2100 37 µg/100ml 6 µg/100ml Urinary 17-hydroxysteroids : 16 mg/24 hrs
12	2808 r in 15 days to sacroiliac region. Good relief of pain	None	Plasma cortisol : 25th May '72† 5th June '72† am 25 µg/100ml pm 30 µg/100ml Urinary 17-oxosteroids : 6 mg/24 hrs 3.3 mg/24 hrs Urinary 17-hydroxysteroids : 13 mg/24 hrs 5 mg/24 hrs
20	None	None	Urinary 17-oxosteroids : 18 mg/24 hrs Urinary 17-oxogenic steroids : 17 mg/24 hrs Plasma cortisol : am 11 µg/100 ml pm 19 µg/100 ml Urinary free cortisol : 220 µg/24 hrs.

* For surgical details see Table 7. III.

† Postoperative results

further 3 patients (Cases 6,7 and 9) a diagnostic biopsy only was taken. The diagnosis was established at autopsy in 2 patients (Cases 3 and 17), in 1 of whom a prior liver biopsy had revealed "metastatic tumour".

Adequate details of therapy are available only for 13 patients (Table 7.II). Five of the patients received post-operative radiotherapy to the tumour bed; the dose range utilizing megavoltage equipment was between 2040r and 4800r over 21 to 52 days (Table 7.II). There was no significant clinical change in these 5 patients with the exception of Case 11 (vide infra). Two patients (Cases 10 and 12) received palliative radiotherapy for bone metastases with relief of pain. One patient (Case 6) was treated by radioactive-gold pituitary ablation with no measurable tumour response. Four other patients received no radiotherapy (Table 7.II).

Four patients were treated with chemotherapeutic agents. Two (Cases 2 and 12) received in addition to radiotherapy, o,p'-DDD (2-chlorophenyl)-2-(4-chlorophenyl)-1,(1-dichloroethane), 8-10g per day. Case 12 has shown good tumour regression in the positive nodes as judged by lymphangiography. Case 1 received methotrexate, 10mg per day, and the final patient (Case 11) was treated with cyclophosphamide (0.5-1mg intravenously) weekly, vincristine sulphate (0.5mg intravenously,) methotrexate (50mg intravenously), and 5-fluorouracil (5-FU; 300mg intravenously) singly or in combination at various time intervals after completing a course of radiotherapy. After two doses of 5-FU she developed a duodenocolic fistula which was surgically repaired. No tumour remained in the treated area, and while recovering from surgery and without receiving further chemotherapy,

her lung metastases also completely disappeared radiologically. This remission, however, was short-lived.

Three patients received neither radio-nor chemotherapy postoperatively and one of them (Case 4) was known to be alive 18 years after operation.

B. RESULTS

1. Survival

Adequate data are available in 18 cases (Table 7.III) one of whom was an autopsy diagnosis. Six patients died within 6 months of diagnosis. Two patients survived 7 months, one 13 months and two, 7 years. At the end of the present survey, two patients were alive and well without evidence of recurrent disease (Case 4, 18 years; Case 20, 1 month). Four patients were alive but had evidence of remaining or recurrent tumour (Case 12, 5 months; Case 14, 3½ years; Case 18, 4 years; Case 19, 6 months).

2. Pathology

The details of the pathological features are shown in Table 7.III. All the tumours are large, lobular, and with cut surfaces which are reddish-brown in colour and show areas of recent and old haemorrhage and evidence of necrosis (Fig 7.1). The tumours have a thin capsule which may be adherent to the adjacent tissues. They weighed from 700-4500g and measured up to 40 X 33 X 25cm.

A variable histological pattern is seen not only between tumours but also in the same lesion. The tumour cells arranged in sheets, large alveoli, or cords which may radiate from the

Table 7.III. Pathological features of 20 patients with non-hormonal adrenocortical carcinoma.

Case number	Macroscopic features		
	Size	Surgery	Autopsy
1	10 X 7.5 cm	Right adrenal tumour removed –	Cerebral haemorrhage Metastases to brain and ribs
2	Not known	Right adrenal tumour infiltrating liver, aorta, surrounding tissues; partially removed	Not performed
3	16 cm diam	Needle biopsy of liver showed metastatic carcinoma. Inoperable left adrenal tumour seen at laparotomy	Invasion and obstruction of renal and adrenal veins and inferior vena cava. Metastases to liver and kidney
4	25 X 20 cm	Partial removal of cystic tumour filling entire upper right quadrant displacing stomach, duodenum, colon, kidney	
5	"Foetal skull"	Right adrenal tumour removed from between liver and diaphragm: ruptured during removal	Metastases to liver
6	"Large"	Tumour arising from left of liver and surrounding spleen: metastases to liver; inoperable	
7	25 X 19 X 15 cm	Inoperable right adrenal tumour extending beyond capsule involving perirenal tissues, liver, lymph nodes	Metastases to left kidney and peritoneum

Histological features				Histological degree of malignancy	Survival (years)
Cell		Pleomorphism			
Arrangement	Type	Cellular	Nuclear		
Lobulated sheets radiating cords	Compact	Marked	Marked	High	2/12
Lobulated sheets, cord.	Compact	Moderate	Slight	Moderate	13/12
Lobulated sheets, cords	Mixed compact and clear	Moderate	Moderate	Moderate	3 days
Lobulated sheets, radiating cords	Compact	Slight	Slight	Low	Alive 18
Lobulated sheets, few radiating cords	Mainly compact; few clear cell nests	Moderate	Moderate	Moderate	7/12
Sheets, acini, radiating cords	Compact	Slight	Slight	Low	3/12
Lobulated sheets	Compact	Slight	Slight	Moderate	3/12

Table 7. III. (Continued).

Case number	Macroscopic features		
	Size	Surgery	Autopsy
8	Not known	Left adrenal tumour involving renal vein: removed except for renal vein extension	Metastases to lung, liver, vertebral bodies, and femur
9	11 cm diam	Inoperable tumour involving spleen, great vessels, vertebral bodies	
10	20 X 20 X 20 cm	Removal of tumour displacing stomach and pancreas	
11	40 X 33 X 25 cm	Right adrenal tumour removed	
12	30 cm 4. 5 Kg	Removal of right adrenal tumour	
13	Not known		
14	27. 5 X 22 X 12. 5 cm	Removal of left adrenal tumour adherent to adjacent organs. Peripheral fleshy tissue: central necrosis	
15	15 X 15 X 9 cm	Left adrenal tumour removed with adherent spleen, kidney, tail of pancreas: capsule intact. Central haemorrhagic necrosis	

Histological features				Histological degree of malignancy	Survival (years)
Cell		Pleomorphism			
Arrangement	Type	Cellular	Nuclear		
Lobulated sheets, radiating cords	Compact	Marked	Marked	High	7/12
Lobulated sheets	Mixed compact and clear	Marked	Marked	Moderately high	3/12
Lobulated sheets; few cords	Compact	Marked	Marked	Moderately high	6/12
Sheets, few cords and acini	Compact	Moderate	Moderate	Moderate	6
Sheets, radiating cords	Compact	Slight	Slight	Low	Alive 5/12
Lobulated sheets, radiating cords	Spindle	Marked	Marked	High	Not known
Lobulated sheets, few cords	Compact	Moderate	Moderate	Moderate	Alive $3\frac{1}{2}$
Lobulated sheets, acini, radiating cords	Compact	Slight	Slight	Low	6

Table 7.III. (Concluded).

Case number	Macroscopic features		
	Size	Surgery	Autopsy
16	23 X 20 X 15 cm	Removal of left adrenal tumour. Smooth, encapsulated, fleshy with central necrosis	
17	1100 g		Right adrenal tumour invading inferior vena cava: metastases to lung
18	19 X 17 X 9 cm	Removal of lobulated tumour of left adrenal: left lobectomy for lung metastases	
19	18 X 15 X 13 cm 1520 g	Removal of right, well-encapsulated adrenal tumour adherent to liver and invading adrenal vein	
20	12 cm diam 700 g	Removal of left adrenal tumour with adherent kidney and spleen	

Histological features				Histological degree of malignancy	Survival (years)
Cell		Pleomorphism			
Arrangement	Type	Cellular	Nuclear		
Lobulated sheets, radiating cords, acini	Mainly compact: nests of clear cells	Slight	Slight	Low	Not known
Lobulated sheets, few radiating cords, acini	Mainly compact: few clear cells	Moderately severe	Moderately severe	Moderate	Autopsy diagnosis
Trabeculated sheets, radiating cords	Mixed compact and clear	Moderate	Moderate	Moderate	Alive 4
Lobulated sheets radiating cords	Compact	Slight	Slight	Low	Alive 6/12
Lobulated sheets	Mixed compact and clear	Marked	Marked	High	Alive 1/12

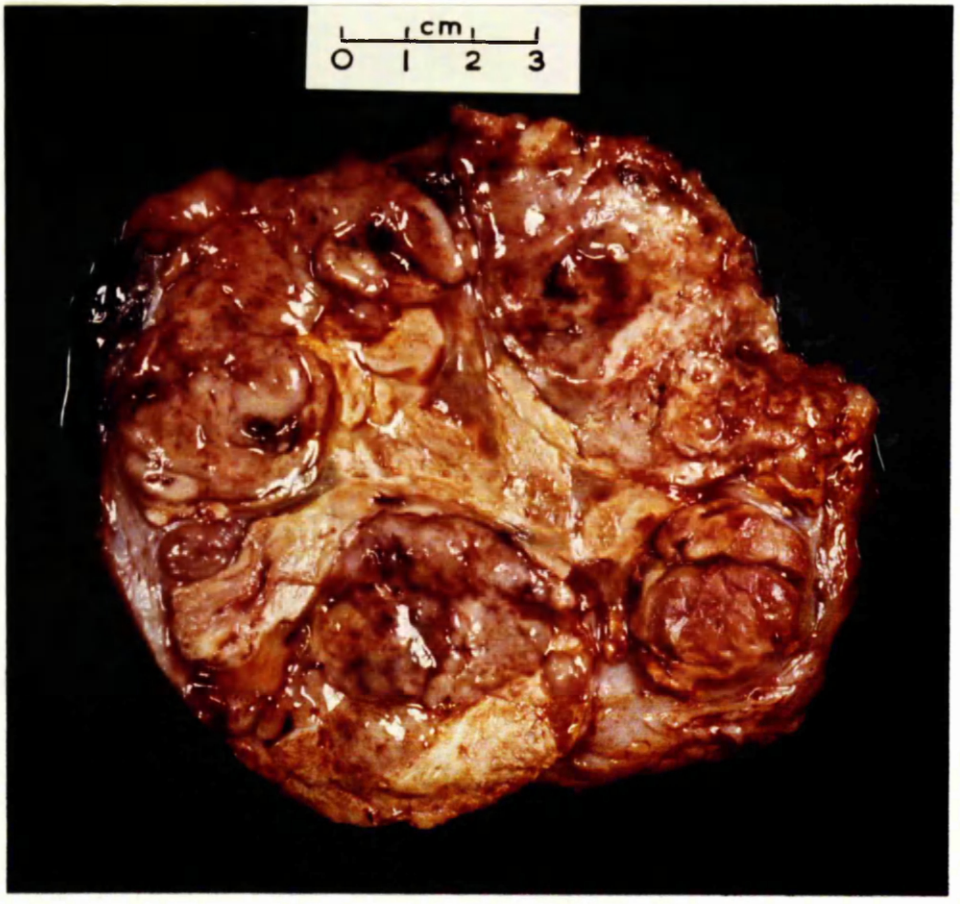


Fig. 7.1. Colour photograph showing the cut surface of an adrenocortical carcinoma from case number 20. This 12 cm. tumour weighed 700g. Areas of haemorrhage and necrosis can be seen clearly. (Magnification X 0.83)

numerous thin-walled vascular sinusoids (Figs. 7.2 & 7.3) can vary in size from normal to large bizarre forms, but are generally polygonal with abundant eosinophilic lipid-poor cytoplasm,and resemble the cytoplasmic features of "compact cells" seen in the zona reticularis of the normal adrenal gland. Small groups of lipid-laden clear cells are also seen occasionally.

The nuclei are usually large, single, and vesicular with one or more prominent nucleoli (Fig.7.3); giant bizarre or multinucleate forms are not uncommon. Nuclear and cytoplasmic pleomorphism may be marked in some tumours and slight in others. Several tumours exhibit different degrees of pleomorphism in adjacent parts (Fig. 7.4). Mitotic activity varies from 3 per HP field to less than 1 per 10 HP field.

We used those histological features - cellular and nuclear pleomorphism, nuclear vesicularity and mitotic activity - of proven value in assessing the malignant potential of endocrine tumours (Neville & Symington, 1967; Symington, 1969) and graded the present series of tumours into those of high, moderate or low malignancy. Those classified as highly malignant died within 7 months, those of moderate malignancy between 1 month and 6 years, while those of low malignancy survived up to 18 years postoperatively, although one died within 3 months (Table 7.III). Thus, as a general rule, using those criteria, the higher the grade of histological malignancy, the shorter the survival (but exceptions exist).

The adrenal cortex attached or contralateral to the tumour was available for study from only four patients (Cases 5,8,10 and 17). The appearances were within normal limits (Fig. 7.5) in both cases (Cases 10 and 17) where the gland was not compressed.

Fig. 7.2. Photomicrograph of adrenocortical carcinoma showing cords of tumour cells radiating out from a central vascular sinusoid. (H & E X 380)

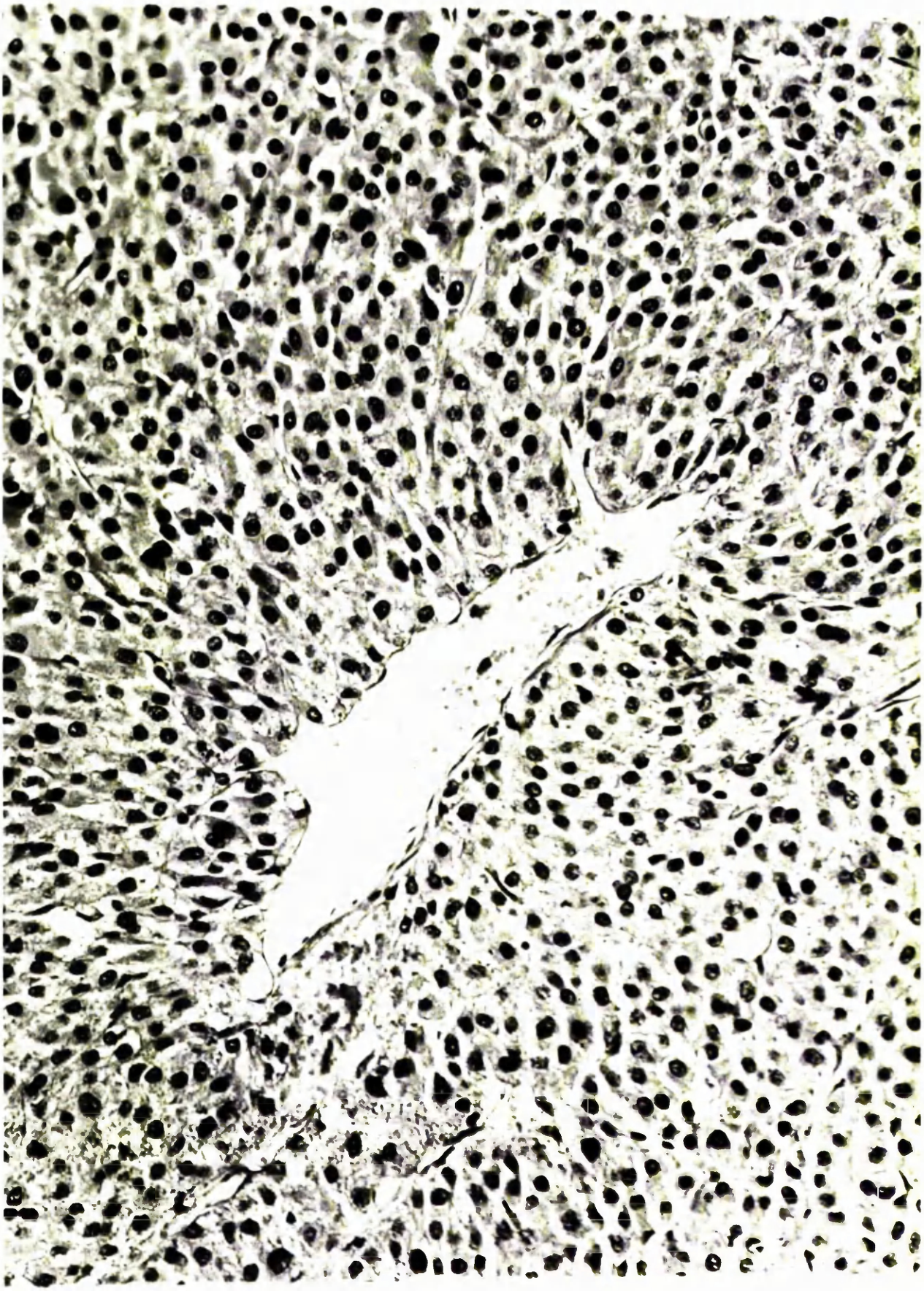


Fig. 7.2.

Fig. 7.3. Photomicrograph of adrenocortical carcinoma showing general arrangement of cells forming large alveolar structures. The nuclei are large and vesicular with a prominent nucleolus. (H & E X 380)

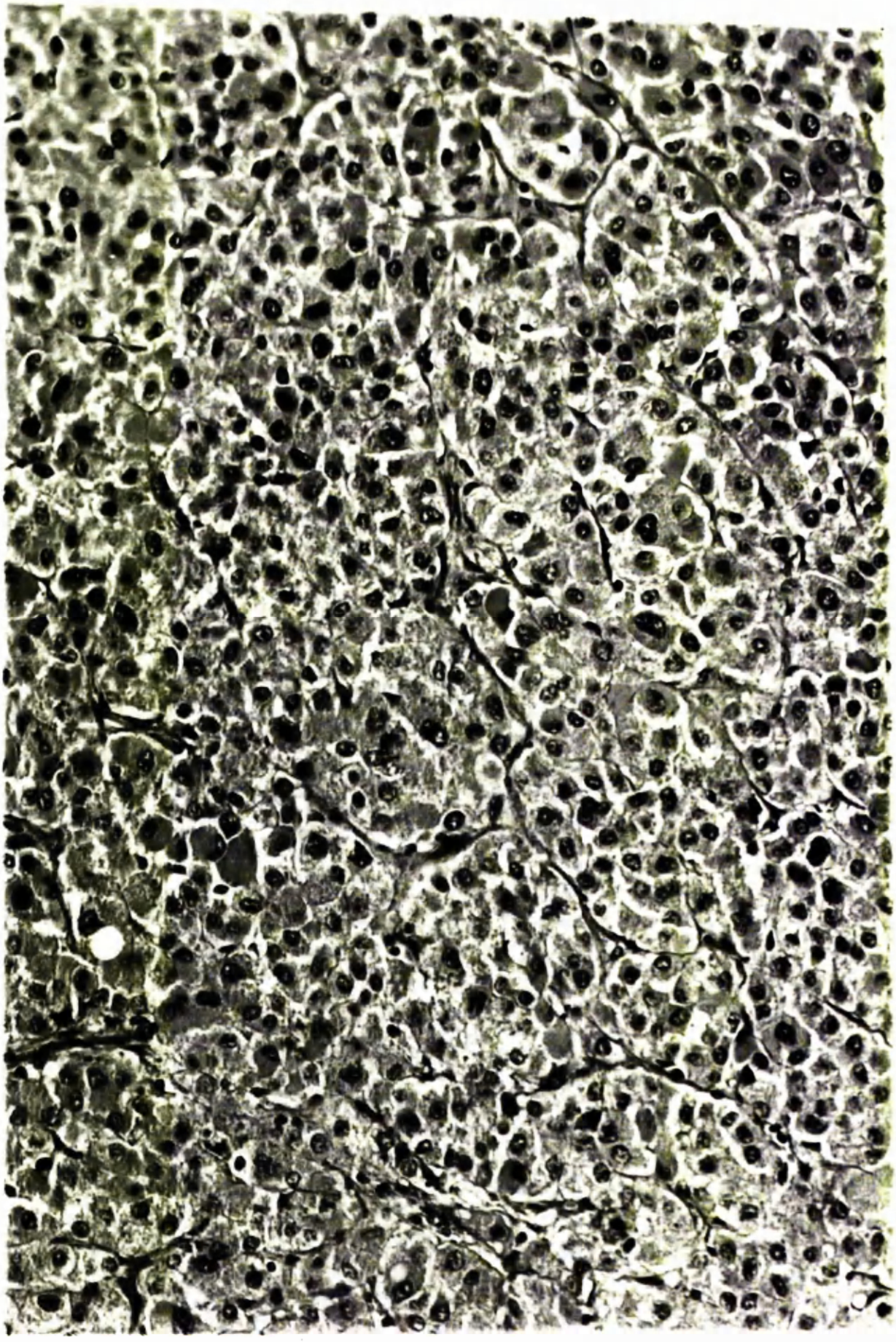


Fig. 7.3.

Fig. 7.4. Photomicrograph of adrenocortical carcinoma showing different degrees of cellular and nuclear pleomorphism in adjacent fields. The upper right segment contains small, rounded, fairly uniform cells in contrast to the lower left field where the cells are variable, some being very large and bizarre. (H & E X 380)

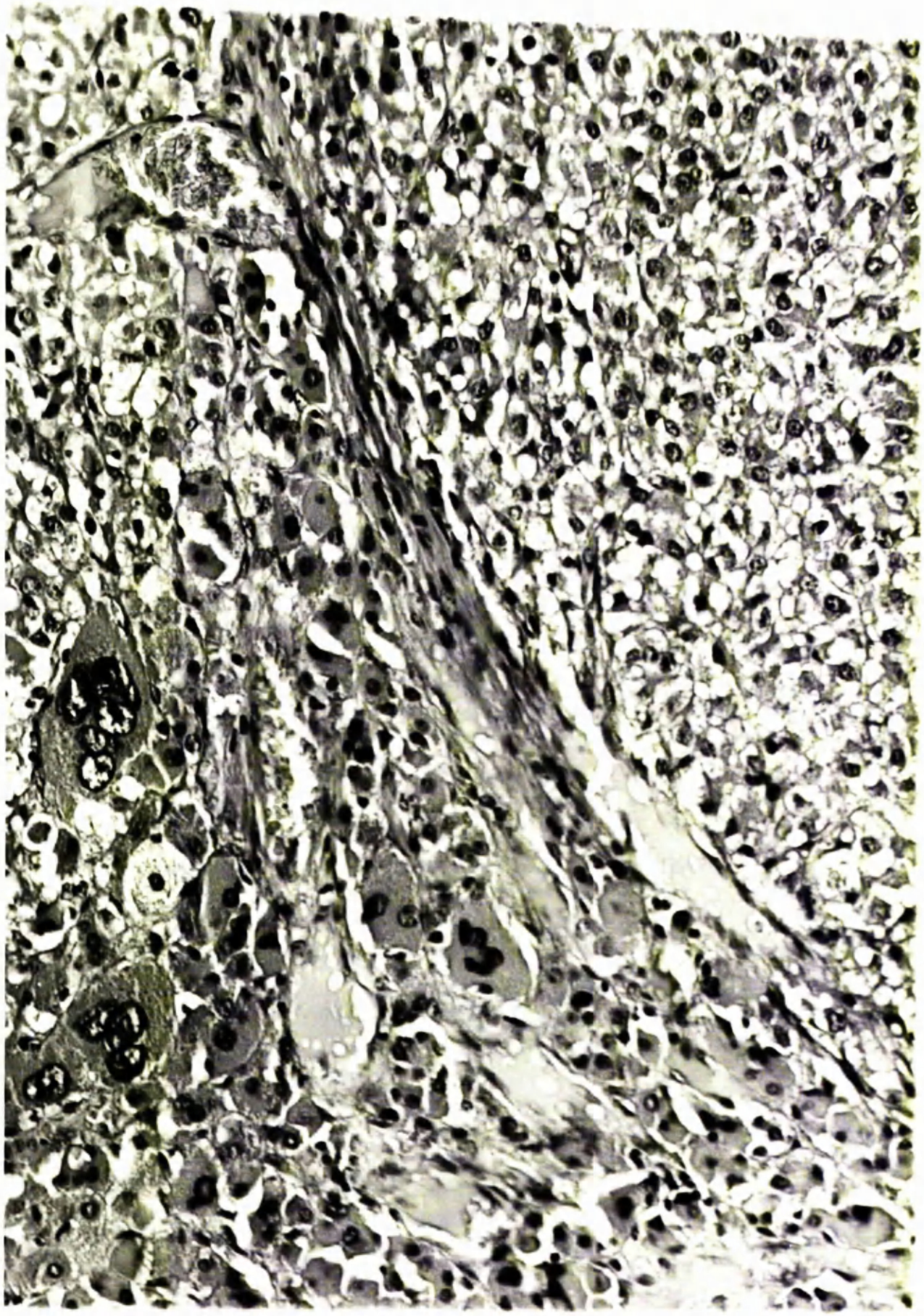


Fig. 7.4.

Fig. 7.5. Photomicrograph showing uninvolved adrenal cortex attached to the tumour in Case 10. The clear and compact zones, and the zona glomerulosa are present in the normal proportions. (H & E X 285)

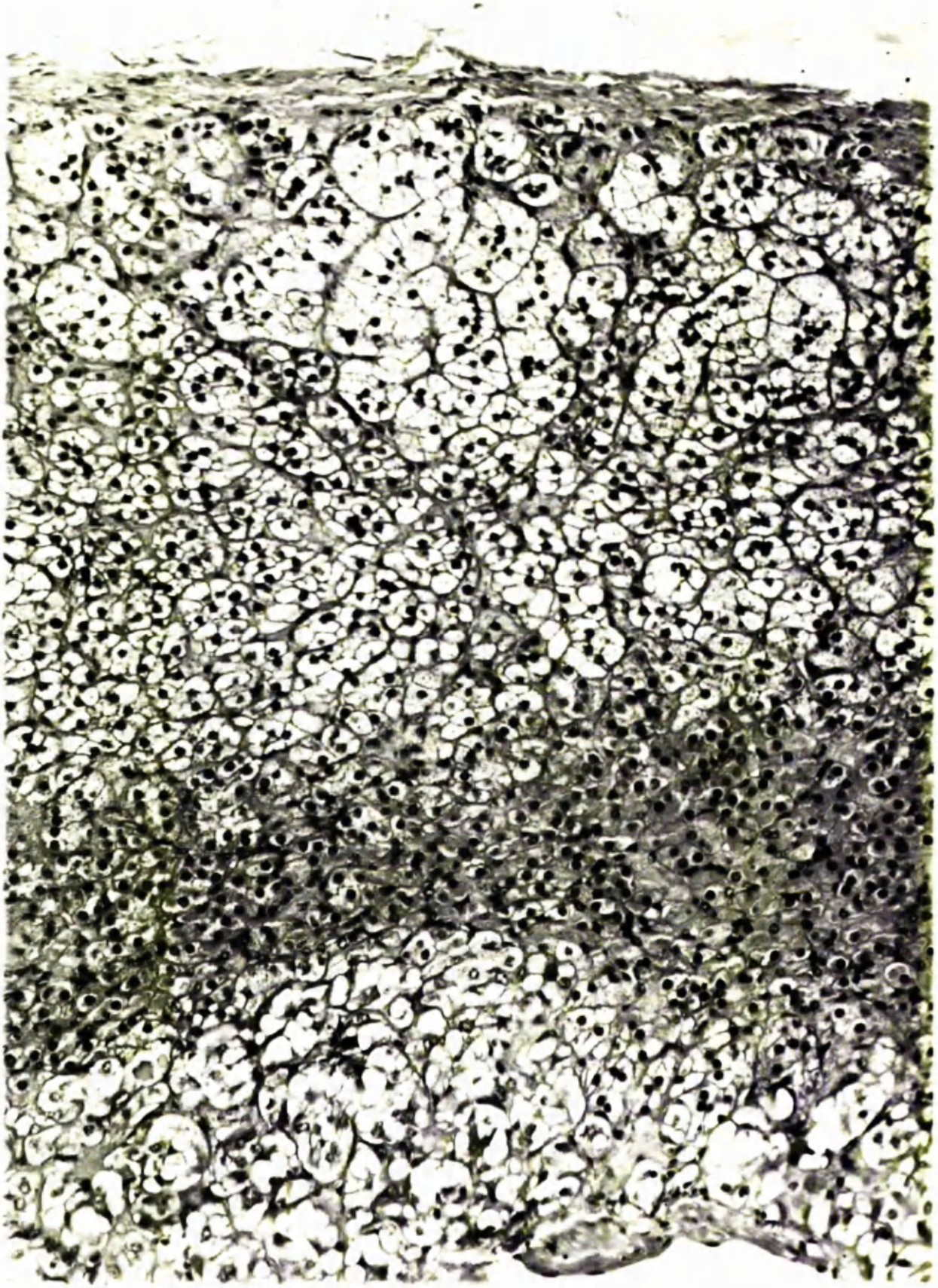


Fig. 7.5.

Evidence of tumour spread was noted in many cases. Two of the tumours directly invaded local structures, and in six, there was extension along the central adrenal or renal veins, or involvement of the inferior vena cava or aorta (Table 7. III). The local lymph nodes were involved in only two cases (Table 7.III); distant metastases were observed initially or developed in 14 patients, the commonest sites being liver (6 cases), bones (6 cases), lung (5 cases), and brain (2 cases).

C. DISCUSSION

One hundred and fifty-eight cases with non-hormonal adrenocortical carcinomas have been collected from the literature (See References; articles marked with "R") and are now reviewed together with the present series, making a total of 178 examples. This tumour, thus, is extremely rare, an observation which is further demonstrated by the fact that members of our department have personally seen and reviewed during the past 12 years the pathology of 112 adrenal tumours causing hypercorticalism (Neville & Mackay, 1972; Neville & Symington, 1966, 1967; Symington, 1969).

1. Age, sex, incidence and location of tumours

In 133 of the 178 patients, age data are known and are shown in Fig. 7.6 and Table 7.IV. There appear to be two distributions, one between 0-10 years and the other between the 5th and 7th decades (Fig. 7.6) (Craig & Landing, 1951; Sherman, et al., 1958).

Fig. 7.6. Age and sex distribution of 133 patients with non-hormonal adrenocortical carcinoma described in the literature and in the present series.

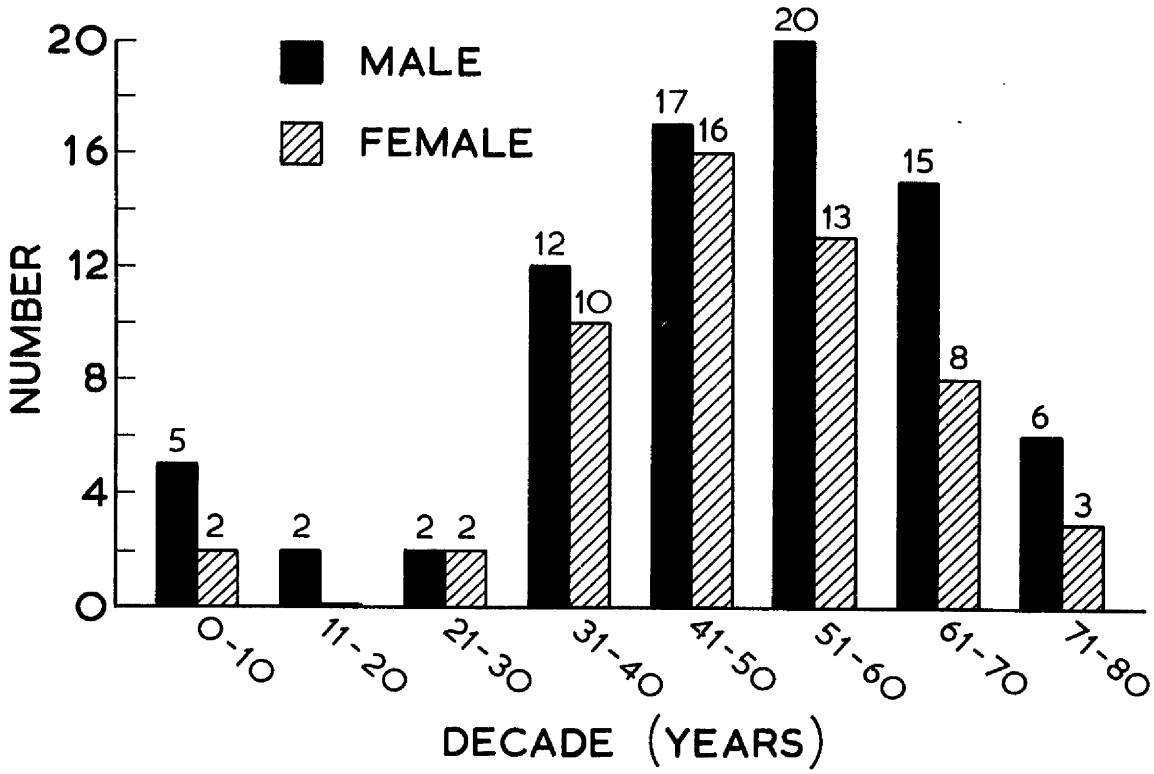

MALE
FEMALE
NUMBER
DECADE (YEARS)
0-10
11-20
21-30
31-40
41-50
51-60
61-70
71-80
0
4
8
12
16
20
5
2
2
2
2
12
10
17
16
20
13
15
8
6
3

Fig. 7.6.

Table 7. IV. Sex distribution and incidence of presenting signs and symptoms of 172 patients with non-hormonal adrenocortical carcinoma described in the literature.

		Literature	Present series	Total
Sex distribution	Male	100	11	111
	Female	52	9	61
	No information	6		6
Signs and symptoms	Pain	49	11	60
	Mass	68	11	79
	Weakness/anorexia	38	9	47
	Fever	24	1	25

Males are affected more often than females (Table 7.IV). In 89 of the cases in the literature in which the site is stated, the left adrenal was involved in 55 and right in 30. In the remaining 4, tumour involved both adrenal glands. It is not known if these represent separate primary tumours or whether one is a metastasis of the other.

2. Signs and symptoms

Table 7.IV records the four commonest presenting signs and symptoms. In their discussions; Wood et al. (1957), Lipsett et al. (1963), and Heinbecker et al. (1957) stated that the clinical onset was insidious and it may be months and sometimes years before a diagnosis is made, by which time a mass is palpable or the pain is severe. A left-sided tumour may mimic a gastric ulcer or tumour; gastro-intestinal bleeding was a presented symptom in six cases. The lack of early symptoms allows the tumour to remain undetected. Therefore, patients may present initially in bizarre fashions such as simulating the Budd-Chiari syndrome (Dupuy et al., 1964) with a right adrenal tumour, acute perforated appendicitis resulting from a metastasis (Grendahl, 1958), visual problems due to a secondary deposit in the retinal choroid (Francios et al., 1952), or pelvic complaints due to vaginal metastases (Hirsh-Hoffman, 1928).

Fever was a feature in all cases described by Wood et al. (1957) but in only 16 other cases in the literature. In our series, only one case, a child of 2½ years, had a fever. Its significance is obscure; fever may decline immediately after surgical removal of the tumour (Weiss et al., 1970) and has been related to the presence of marked tumour necrosis (Vandergrind et al 1964; Weiss et al., 1970; Wood et al., 1957).

3. Diagnostic radiological studies

The most valuable diagnostic radiological test is the intravenous pyelogram; 22 out of 24 carried out by other workers and 12 out of 13 in our series being positive, and therefore should be the first study performed if the diagnosis is suspected. Arteriography is helpful in outlining its vascular supply and may detect involvement of the contralateral gland. Presacral air insufflation can outline the mass more clearly but gives no further information as to its nature.

Due to the rarity of this type of tumour, the role of lymphangiography remains to be established. The adrenal gland is rich in lymphatics which drain to the para-aortic nodes (Ackerman & Del Regato, 1970). Following adequate surgery a lymphangiogram may not only detect occult disease in the lymph node chains but may also provide a visual guideline for further radio- or chemotherapy.

4. Pathology

The tumours of the present series have macroscopic appearances similar to those of most functioning adrenocortical carcinomas described in the literature (Mackay, 1969; Neville and Mackay, 1972; Neville & Symington, 1966,1967; Symington, 1969) but are generally much larger, probably because they do not produce clinical symptoms at an early stage. The predominant cell is compact in type, a feature not emphasized by other authors. Indeed most tumours described in the literature have been purported to have had more clear than compact cells, a feature which is at variance with our experience of all other large adrenal tumours which cause hypercorticalism with the exception of those causing hyperaldosteronism. A possible reason for the cytoplasmic differences is

discussed later.

Some authors (Heinbecker et al., 1957; Huvos et al., 1970; Birke et al., 1959) have suggested that non-hormonal tumours have peculiar features not shared by functioning tumours. We failed to detect any single histological feature, or group of features, which enabled definitive histological assessment of the functional capacity of those adrenocortical carcinomas, and their differentiation from other compact cell carcinomas causing hypercorticalism. However, examination of the attached or contralateral cortex will give some clue as to the secretory potential of a tumour causing Cushing's syndrome when it shows cortisol-induced atrophy (Neville & Symington, 1966) or Conn's syndrome when the zona glomerulosa is hyperplastic in association with a gland of normal width (Neville & Syminton 1967). While the presence of distinct tumour spread, e.g. to the lymph nodes or kidney, is pathognomonic of a malignant tumour, such criteria may not be present at the time of initial treatment. Consequently, reliance has to be placed upon the gross pathology and morphology.

It seems that the largest tumours in this series are associated with a better prognosis (Cases 4,11,14,15 and 18) (Table 7.II). However, this criterion is not a reliable guide to management as is shown by Cases 7 and 10 (Table 7.III) who died within 6 months with large tumours. The histological features of capsular invasion or tumour cells in vascular spaces are also quite independent of the potential behaviour of the tumour and reaffirm previous conclusions that these criteria occur with benign and malignant tumours (Neville & Mackay, 1972; Neville & Symington 1967; Symington, 1969). While other features, such as nuclear pleomorphism and vesicularity, which have proved of value

in other studies of endocrine tumours, did give some indication of the "Malignant" potential, such criteria were by no means absolute. Consequently, all such tumours are best viewed as as carcinomas and the patient followed accordingly at frequent intervals when suitable steroids assays should be conducted (vide infra).

5. Metastases

Inadequate data with regard to sites of metastases are recorded in the literature. In his series of 55 examples of "functioning" and "non-functioning" tumours, Macfarlane (1958) reported a 31 percent overall incidence of local invasion with local lymph node involvement in 44 percent and metastases to the liver in 68 per cent, lungs in 47 per cent, and bones in 18 per cent. In our series, the local lymph nodes were involved in 10 per cent of patients, and osseous metastases (30 per cent) were as frequent as those in liver. We found two cases (Cases 1 and 18) of verified brain lesions; five other cases were found in the literature (Ansari & Shafer, 1971; Demaret et al., 1968; Francios et al., 1952; Horn, 1956; Sherman et al., 1958). Previous authors (Hutter & Kayhoe, 1966; Huvos et al., 1970; Lipsett et al., 1963) have stated that cerebral metastases were extremely rare.

6. Steroid assays

Non-hormonal adrenocortical carcinomas are not without steroidogenic activity. Rather, they are incapable of forming excess active hormones, such as cortisol, aldosterone, androgens, or oestrogens.

The work of Fukushima and Gallagher (1963) has shown that so-called "non-functioning" tumours can form pregnenolone, and that its metabolites occur in the urine in the form of pregn-5-ene-3β,20α-diol and pregnane-3α,20α-diol. More recent work by Fantl et al. (1973) has confirmed and extended this to show that 16α-hydroxylated metabolites of pregnenolone may also occur in the urine. The tumours of Cases 3 and 20 were found to have this secretory pattern. Thus, some of these tumours, appear to have enzymatic defects involving the 5Δ-3β-hydroxysteroid dehydrogenase-isomerase systems and possibly the 17α-hydroxylase, which account for failure of pregnenolone utilization (Fig. 7.7). Enzymatic defects could also account for the presence of clear cells in some tumours (vide supra). If there is a deficiency of the C20:22 desmolase system, cholesterol utilization will be prevented and with its esters, it will accumulate in the cytoplasm and give the tumour cells a lipid-laden clear appearance. This is the cause of the appearance of the adrenal in congenital lipoid hyperplasia, a rare form of the adrenogenital syndrome (Prader & Siebenmann, 1957).

Therefore in the follow-up of patients treated for adrenocortical carcinomas, without overt function, it is essential that the urinary metabolites of pregnenolone are studied and used as tumour index substances to gauge the clinical status of the patient rather than the typical adrenal steroid products.

7. Treatment

The primary role of treatment remains surgery, which was carried out on 57 out of 65 patients recorded in the literature.

Fig. 7.7. Schematic representation of main steroidogenic pathways. Recommended systematic nomenclature of steroids and enzymes is given in Appendices I and II.

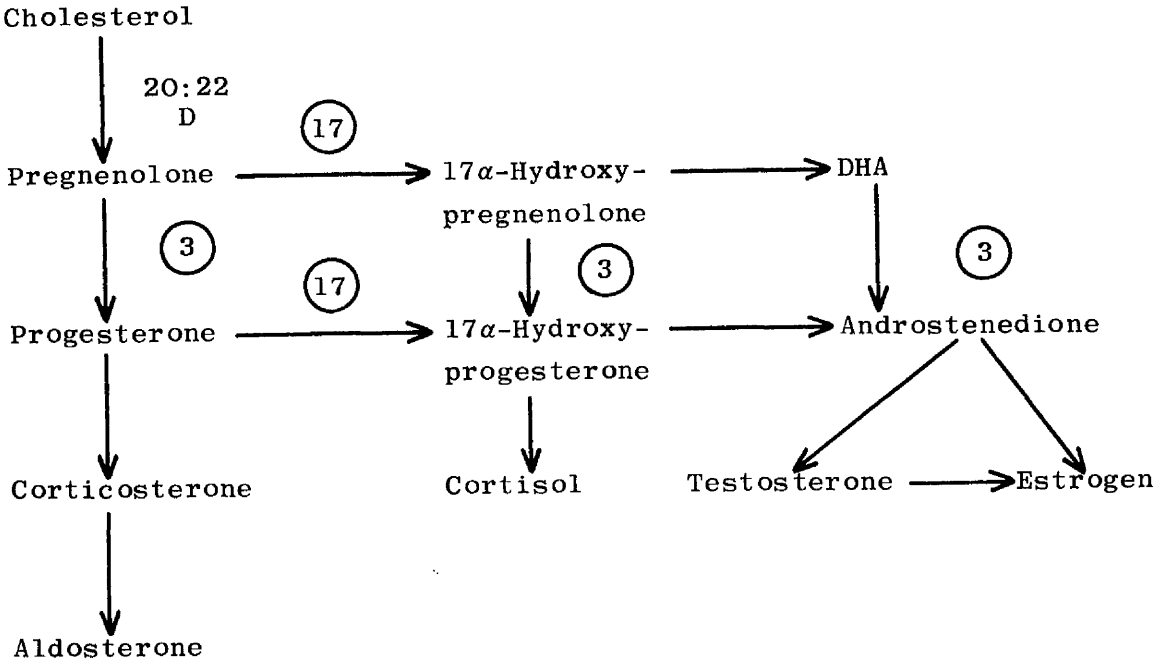


Fig. 7.7. Steroid biosynthetic pathways.

20:22D	C20:22 desmolase
17	17α-hydroxylase
3	Δ^5-3β-hydroxysteroid dehydrogenase-isomerase systems

While others (Lipsett et al., 1963) claim that this tumour is radioresistant, we found that high doses of external irradiation may be beneficial (Cases 10-12). In our hands, a response to radiotherapy indicates that the tumour may also respond to chemotherapy.

Hutter and Kayhoe (1966a&b) and Bergenstal et al. (1960) have described the oral administration of o,p'-DDD for the treatment of adrenocortical carcinomas with prednisone being given concomitantly if signs of adrenal insufficiency are suspected. Gastro-intestinal toxic manifestations (diarrhoea, nausea, and vomiting) develop in 83 per cent of patients, neurological symptoms (somnolence and lethargy) in 41 per cent and skin rashes in 14 per cent. Other effects such as dizziness, muscle tremor, headache, confusion, and weakness have also been noted. The control of these symptoms is achieved by reducing the total dose or by giving the drug intermittently.

Because responses to o,p'-DDD occur slowly, treatment should be continued for at least 8 weeks provided the side effects are not too severe. The dramatic but short lasting response to combination chemotherapy in addition to radiotherapy in one patient (Case 11) suggests that this form of therapy may be more effective than o,p'-DDD alone.

In view of these findings, it seems worthwhile to propose that treatment might be attempted in a manner similar to that employed for other highly malignant tumours, namely surgery, radiotherapy and combination chemotherapy.

8. Prognosis

This tumour carries a poor prognosis. Of 61 patients on whom survival data are available, 42 were dead within 1 year,

and of those 24 died within the first 2 months. No correlation was found between age at diagnosis or sex and the survival. The only long term (18 years) survivor in the literature is presented in the series.

9. Conclusion

For non-hormonal adrenocortical tumours surgery remains the treatment of choice, with radical excision whenever feasible, even if this necessitates the removal of adjacent adherent organs, e.g. kidney, or spleen. Intravenous pyelography and arteriography are the best aids to diagnosis. The functional capacity of these tumours must be assessed before surgery, and we recommend that the following should be measured: (A) 24 hours urinary 17-hydroxy- and 17-oxosteroids and steroid metabolites of pregnenolone; (B) morning and evening plasma cortisol levels; (C) response to ACTH stimulation and (D) dexamethasone suppression.

Postoperatively, the presence of lymph node involvement may be assessed by lymphangiography. We have found radiotherapy to be useful in the treatment of residual tumour and metastases. While chemotherapy with o,p'-DDD is of limited value, a combination of other chemotherapeutic agents may give more beneficial results. The course of the disease should be monitored by serial urinary steroid metabolite studies.

SECTION III : GENERAL DISCUSSION AND SUMMARY

CHAPTER 8

GENERAL CONCLUSIONS ON FUNCTIONAL AND MORPHOLOGICAL CORRELATIONS

CHAPTER 8

GENERAL CONCLUSIONS ON FUNCTIONAL AND MORPHOLOGICAL CORRELATIONS

The general theme of this thesis has been the correlation between function and morphology in normal and neoplastic tissues. Morphological changes have been examined at histological and cytological levels. These morphological changes are well known, and in most cases a functional interpretation has been made although not fully ratified by experimental proof.

Jost (1953) in his review of his pioneering work on the function of the mammalian foetal testis clearly demonstrated that this organ functions *in utero*, and is required for normal sexual development of the male foetus. This functional activity coincides with the morphological appearance of the interstitial cells of Leydig which are known to produce androgens in the adult testis. The foetal testis also secretes androgens (Winter *et al.*, 1977) which are required for normal development of the male genital ducts (Jost, 1953). The Mullerian inhibitory factor (MIF) is also synthesised and released by the foetal testis, and production of this compound occurs at a time when Sertoli cells and Leydig cells are both present. The morphological basis of MIF production is not yet certain. Is it produced by Sertoli cells, Leydig cells, both or neither? Morphological studies alone cannot answer this question.

The histochemical studies of Baillie and his co-workers (See Chapter 1) have shown that steroidogenic enzymes are present when MIF is produced.

However, the substrate specificity of these enzymes changes during the period of sex duct differentiation although this functional change is not reflected in definite morphological alterations in the steroid-producing cells of the testis. Therefore, the steroidogenic activity of Leydig cells cannot be assessed morphologically.

The Sertoli cells of adult testes produce an androgen binding protein (ABP) which binds testicular androgens before they reach the circulation. MIF is not a steroid in free form and at least part of it is a protein, probably produced by the foetal Sertoli cells (Josso et al., 1977). If this protein is similar (or identical) to ABP then it is logical to assume that MIF is a protein requiring the addition of a steroid molecule before it is biologically active. If so, is this steroid progesterone?

On theoretical grounds, progesterone seems to be a likely candidate (See Chapter 1). This thesis has demonstrated experimentally that progesterone has a deleterious effect on the Mullerian duct in organ culture although this effect has not been proved to be specific. The in vitro assay system used for detecting MIF is a bioassay with a morphological end-point (Picon, 1969). Jost (1953) has already described the pitfalls of such a system when he demonstrated that DOCA dissolved in olive oil containing benzyl alcohol caused regression of the Mullerian duct. The initial conclusion that DOCA was MIF had to be discounted when the Mullerian inhibitory effect was found to be due to the olive oil/benzyl alcohol solvent system used.

The morphological end-point of this bioassay is therefore non-specific thus accentuating the difficulties of making a direct functional interpretation of morphological changes.

Non-specific regression of the Mullerian duct also occurs in response to certain prostaglandins (Josso, 1974a), and the experiments presented in this thesis demonstrate that 1% ethanol also results in non-specific Mullerian duct inhibition as a result of generalised cytoxicity.

Progesterone causes Mullerian duct regression in organ culture, but how specific is this effect? Is this another example of non-specific cytoxicity; or an example of one biological substance mimicking the effect of another; or is it a reflection of a physiological process indicating that progesterone acts on the Mullerian duct *in vivo*? Clearly, the bioassay system used is not sufficiently specific to answer those questions, and more evidence must be accumulated before a physiological role for progesterone in this context can be assumed.

Further evidence for a possible physiological role of progesterone came from the monolayer culture experiments described in this thesis. Foetal rat testes in monolayer culture produce and secrete progesterone, and this activity is stimulated by cAMP. Does this indicate a biological role of foetal testicular progesterone?

Monolayer cultures of human adult and prepubertal testes produce progesterone (O'Hare *et al.*, 1976a) as do monolayer cultures of adult rat testes (Khatim & O'Hare, 1976). Testes from patients with testicular feminisation also produce progesterone in monolayer culture (Grigor, Bullman & O'Hare, unpublished observation; *vide supra*). Shin (1967) demonstrated that a monolayer culture derived from an interstitial cell tumour produces progesterone *in vitro*, and Channing (Channing, 1969a&b; Channing & Grieves, 1969) described "spontaneous luteinization" of ovarian granulosa cells in culture, a process whereby the cells *in vitro* lose the ability to secrete oestrogens and they secrete progesterone instead.

Some steroid-producing cells continue to produce androgens *in vitro* as O'Hare *et al*. (1976a) demonstrated using adult human adrenocortical cells, but there seems to be a general tendency for "spontaneous luteinization" of testicular and ovarian steroidogenic cells in tissue culture (O'Hare *et al*., in press). This tendency casts doubts on the physiological significance of progesterone secretion by foetal rat testicular cells in the monolayer cultures described in this thesis. Do rat testes secrete progesterone *in vivo*, or is there a process of "spontaneous luteinization" of these cells once they are in tissue culture?

Monolayer cultures of endocrine glands are useful in the determination of the exact cellular environment required for normal synthetic activity. Adrenocortical cells in monolayer culture may function normally, but cell-to-cell relationships and the presence of ACTH are important factors in the maintainence of normal function (O'Hare *et al*., 1978). Testicular steroidogenic cells are more demanding in their requirements, and culture methods employed at the present time predispose to a diminution in testosterone production and a loss in responsiveness to HCG.

Foetal rat testicular cells produce progesterone for a short time in non-stimulated monolayer cultures. Reactivation of steroidogenesis *in vitro* by cAMP results in progesterone secretion before testosterone output is detected. Foetal rat genital tracts in organ culture respond to progesterone which causes Mullerian duct regression. These three facts have been demonstrated emperimentally for the first time in this thesis, and point to a possible role of progesterone in ths physiological inhibition of the Mullerian ducts in the male foetal genital tracts. However, definite proof is still lacking.

Progesterone may be involved in Mullerian inhibition *in vivo*, and if so, probably does not act alone. It is more likely to be transported from the foetal testis to the adjacent genital tract by a carrier protein similar to the androgen binding protein produced by the adult testicular Sertoli cells. Morphological studies alone cannot resolve this question, and further functional studies are required before a stronger claim for the role of progesterone can be made.

O'Hare *et al*. (1978) stressed the importance of cell-to-cell relationships and cellular contact in the control of steroidogenesis in adrenocortical cells in monolayer culture. The cell density and the presence of ACTH in the culture medium are important: the presence of ACTH enhances steroid 17 hydroxylation whereas increasing cell density stimulates 11 β-hydroxylase activity. Having established the functional properties of normal adrenocortical cells in monolayer culture, O'Hare and his co-workers examined cultures of neoplastic cells and determined their functional divergence from non-neoplastic cells. The functional differences between normal and neoplastic cells in culture were correlated with their morphological differences in histological sections. The importance of correlating morphology with function in the human adrenal cortex lies in the differentiation of neoplastic and non-neoplastic swellings, and in neoplastic conditions it is important to differentiate between benign and malignant growths.

Non-neoplastic discrete masses in the adrenal cortex are usually indicative of nodular hyperplasia, and this may be associated with Cushing's syndrome if there is excessive trophic stimulation causing increased glucocorticoid secretion.

Hyperplastic nodules are usually multiple although their multiplicity is not always readily apparent. Macroscopically and histologically an apparently solitary hyperplastic nodule and an adrenocortical neoplasm may appear very similar (Neville & O'Hare, 1978; Neville & Symington, 1967). In Cushing's syndrome, therefore, a functional interpretation of the morphological features of the attached adrenal cortex is useful in the differential diagnosis. If the attached cortex is hyperplastic, the lump is a nodule (Neville, 1978). If the attached cortex is atrophic, the lump is a neoplasm (Neville, 1969). The difference is due to the autonomy of the neoplasm.

Neoplasms of the adrenal cortex show varying degrees of autonomy and responsiveness to trophic stimulation. Being autonomous, excessive steroid production occurs causing inhibition of the hypothalamus and pituitary, therefore there are low levels of circulating ACTH. This results in atrophy of the uninvolved adrenal cortex. The autonomous functional activity of adrenocortical neoplasms *in vivo* is reflected by their activity in monolayer culture, and it is found that *in vitro* activity closely resembles *in vivo* activity. There are differences between benign and malignant neoplasms *in vivo*, and these differences can also be examined *in vitro*.

Adrenocortical adenomas are diagnosed early when they are still small tumours because their autonomous production of active steroid hormones cause early clinical symptoms. In tissue culture, their autonomy is retained; however, they also respond to ACTH which stimulates increased steroid output (O'Hare *et al.*, 1978). Adrenocortical carcinomas differ from adenomas in monolayer culture in that an abnormal profile of steroids is produced, and steroid output per cell is reduced.

In addition, they show no response to ACTH stimulation (O'Hare et al., 1978). This has clinical significance because clinical symptoms are less severe resulting in delayed diagnosis. Therefore, malignant neoplasms are usually larger and more advanced at the time of diagnosis.

The differences between benign and malignant neoplasms in tissue culture may have practical importance in the diagnosis of malignancy because the histological criteria of malignancy in adrenocortical neoplasms are ill-defined (Symington, 1969). It may be that all adrenocortical tumours should be examined in tissue culture in order to differentiate between benign and malignant growths.

The functional activity of adrenocortical neoplasms is reflected in the morphology of the adjacent uninvolved gland. An atrophic gland, as already discussed, indicates autonomous production of glucocorticoids by the neoplasm and this may be associated with Cushing's syndrome. In Conn's syndrome (hyperaldosteronism with a low plasma renin), a rather paradoxical situation occurs. The usual presentation is hypertension, and the majority of cases is due to an adrenocortical adenoma with characteristic macroscopic and histological appearances (Neville, 1978; Neville & Symington, 1966). Rarely, malignant aldosterone-producing tumours of zona glomerulosa cells are encountered, and these have a definite histological appearance (Neville & Mackay, 1972; Neville & Symington, 1966) and are readily differentiated from their benign counterparts. The paradox is seen on examination of the adjacent adrenal cortex. These tumours - benign or malignant - have been shown to produce aldosterone (Brode et al., 1962). Although in monolayer culture aldosterone output is not

sustained (Neville & O'Hare, 1978), yet the adjacent cortex usually shows hyperplasia of the zona glomerulosa (Neville & Mackay, 1972; Neville & Symington, 1966) the normal site of aldosterone production.

Hypersecretion of aldosterone should be expected to suppress the zona glomerulosa. Is it possible that these tumours produce an adrenal glomerulosa trophic hormone (AGTH) in addition to aldosterone? Alternatively, the zona glomerulosa may be under constant stimulation due to abnormal production of an AGTH from another source, and after excessive stimulation part of the hyperplastic zone may have undergone neoplastic transformation. Such conjecture is outwith the scope of this thesis. Suffice it to say that aldosterone-producing tumours are usually associated with specific morphological changes in the adjacent adrenal cortex, and in many cases a preoperative distinction between neoplasia and hyperplasia may be made (Ferriss et al., 1970).

Some adrenocortical tumours are associated with a normal-looking adjacent adrenal cortex. This situation arises when the tumour produces sex steroids (Adreno-genital syndrome: Neville, 1969; Symington, 1969) which do not suppress ACTH release; or when the tumour produces very little, or no, hormonally active steroids. The adrenocortical tumours described in this thesis are of the non-hormonal variety. Non-hormonal tumours deviate from normal to a greater extent than hormone-secreting tumours. Therefore, being more dedifferentiated, they would be expected to have a greater tendency to be malignant, and also would be expected to present late when the tumours have reached a large size. The tumours presented in this thesis verify these expectations. The functional capacity of the tumour themselves cannot be assessed on tumour morphology alone, however, the large size

of the tumours and the lack of trophic changes in the adjacent gland indicate a low secretion rate of active hormones.

"Non-hormonal" does not mean "Non-functional". Many tumours which cause no apparent hormonal imbalance have been shown in vivo and in vitro to be associated with production of abnormal steroids. Therefore, an assessment of function requires serum or urine steroid analysis in the tumour-bearing patient. Ideally, the excised tumour should be extracted for steroid content, and steroidogenic activity in tissue culture should be assessed if possible. These methods are required to make a diagnosis of a truly non-hormonal or non-functional tumour. These functional studies have a definite role to play in the management of the patient. Once the profile of steroid secretion has been established, the subsequent course of the tumour can be monitored serologically by measuring the steroids known to be produced by the tumour.

Functional studies are also important in the choice of chemotherapeutic agents. The drug o,p'-DDD has been used in the treatment of patients with adrenocortical carcinomas (Bergenstal et al., 1960; Hutter & Kayhoe, 1966; Molnar et al.,1963) The cytotoxic action of this drug is not fully understood but it probably interferes with the conversion of cholesterol to pregnenolone, and with 11β-hydroxylation of 11 deoxycortisol (Hoffman & Mattox, 1972). If its mode of action depends on steroidogenic activity in the target cells, a truly non-functioning tumour will be resistant to its cytotoxic effects. Since this drug is unpleasant to take (Hoffman & Mattox, 1972) an assessment of tumour steroidogenic activity should be made before subjecting the patient to unnecessary misery.

Carcinomas of the adrenal cortex illustrate the difficulty in correlating morphology with function. The appearance of the adjacent gland gives a clue to the functional capacity of the tumour, but the morphology of the tumour alone gives no indication of its steroidogenic potential. Assessment of malignancy is difficult. Monolayer cultures of adrenocortical tumours give a good indication of *in vivo* activity and in this respect such cultures are more representative than the tissue culture of foetal testes and genital ducts. Functional studies are important in the examination of patients with adrenocortical tumours, and tissue culture studies are also of potential value in the assessment and management of these cases.

Patients with germ cell tumours may also benefit from functional studies. This is demonstrated by the functional aspects of germ cell tumours presented in this thesis paying particular attention to production of AFP by teratomas.

There is a strong correlation between morphology and function of teratomas in that AFP production is often associated with the appearance of yolk-sac elements in the tumour. The histogenetic basis for production of AFP by yolk sac tumours has been presented, and the lack of absolute and exclusive correlation between AFP and yolk sac elements has been discussed. In this part of the thesis, the possible effect of AFP production on the clinical course of the disease will be considered.

Tumours producing AFP tend to run a more malignant course than non-AFP-producing tumours. Production of HCG also appears to be a poor prognostic sign. Are the tumour cells which produce AFP or HCG more malignant than the tumour cells without this synthetic capacity, or do these secretory products have properties

which enhance tumour progression at the expense of the host? Perhaps both these possibilities are true.

HCG is normally produced by the syncytiotrophoblast, a cell which has the capacity to invade blood vessels. If HCG production by a tumour indicates the presence of syncytiotrophoblasts, then early blood vessel invasion and haematogenous dissemination of tumour is to be expected. Similarly, if AFP production is an indication of yolk sac differentiation then the tumour will be expected to have a poor prognosis because of the known highly malignant nature of yolk sac tumours as described by Beilby (Beilby & Todd, 1974; Beilby & Parkinson, 1975) in ovarian tumours.

Many tumours produce HCG even when there is no histological evidence of trophoblastic differentiation (Braunstein et al., 1973b; Gailani et al., 1976), therefore HCG production by testicular tumours need not indicate trophoblastic elements. McManus et al. (1976) also described HCG production by many different types of tumours and suggests that the synthesis of HCG enhanced tumour growth by inhibition of host immunological defence mechanisms. They concluded that the normal presence of HCG in syncytiotrophoblasts may prevent immunological rejection of the foetus and placenta because in vitro studies have shown that HCG is immunosuppressive by preventing lymphocyte transformation (Adcock et al., 1973; Beling & Weksler, 1974; Contractor & Davies, 1973; Han, 1974; Kaye & Jones, 1971). The cellular localisation of HCG may also be important. Hormones are usually stored within secretory granules in the cytoplasm of endocrine cells. However, HCG is exceptional in that it is not only stored in secretory granules, but is also present on the cell surface of syncytiotrophoblasts (Kawarai & Nakane, 1970; Mason et al., 1969; Nakane, 1971; Jenkins et al., 1972).

It may also be situated on the surface of malignant tumour cells (McManus et al., 1976). This surface localisation may protect the placenta and malignant tumours from immunological attack by the host. If so, the presence of HCG production will facilitate tumour progression.

The possible role of AFP in enhancing tumour growth may be considered in the light of the suspected biological properties of this glycoprotein. AFP has been shown to have immunosuppressive properties affecting cell-mediated responses and antibody formation due primary to its interaction with T cells (Dattwyler et al., 1976; Goeben & Thompson, 1976; Tomasi, 1977; Zimmerman et al., 1977). Rat and mouse AFP, and a small proportion of human AFP, bind oestrogens (Tomasi, 1977) and this results in inhibition of oestrogen metabolism (Aussel & Masseyeff, 1976). AFP is also though to have a regulatory effect on cell proliferation and growth (Tomasi, 1977). The immunosuppressive effect of AFP and its regulatory effect on cell kinetics are related to its oestrogen binding (Keller et al., 1966; Tomasi, 1977) and its immunosuppressive properties are dependent on the presence of sialic acid residues (Zimmerman et al., 1977).

Does the immunosuppressive effect of AFP have physiological and biological importance? There is non-specific depression of immunological reactions in the foetus and newborn, during pregnancy, in some hepatic disorders, and in some malignant diseases. Tomasi (1977) and Dattwyler et al. (1976) suggest that this effect is mediated by AFP. Tomasi (1977) also states that in some cancer-prone families, circulating cells of the lymphoid series contain surface AFP although the serum AFP is not elevated. Similar AFP-coated cells are found in some patients with lymphoma.

The immunosuppressive potential of AFP has not gained universal acceptance, and at present the exact biological role of AFP has not been fully established (Sell, 1976). Weller (1976), however, has demonstrated that AFP is required for the survival of chick embryos because antibody to chick AFP causes mortality in the great majority of cases. Sell et al. (1976) tried to demonstrate a similar toxic effect of anti-AFP antibodies on AFP-producing rat tumours. Although some of the tumours showed slight suppression of growth, the majority of tumours were unaffected.

The biological properties of AFP may be important in the survival and progression of AFP-producing tumours. However, these biological properties are not yet fully elucidated and it has been shown in this thesis that the adverse prognostic effect of AFP production by teratomas is not great. It has also been shown that there is not a direct correlation between AFP production and the presence of yolk sac elements just as HCG production does not necessarily imply trophoblastic differentiation. The importance of AFP production lies in the fact that it can be measured readily in the serum of patients with germ cell tumours, and in many cases it serves as an excellent tumour marker. Morphological and functional studies are therefore complimentary in the management of germ cell tumours and both are important in the proper assessment of afflicted patients.

In every aspect of this thesis, it has been shown that morphological studies are incomplete by themselves, and interpretation requires adequate functional studies for verification.

All viable cells are functional. All normal somatic cells in an organism have the genetic capacity of performing all the cellular functions of that organism.

Cells which produce substances for "export" may be exocrine, endocrine or paracrine and these produce effects on the host which may be localised or generalised. These effects are essential for the normal integrated existence of the organism as a whole. The particular function of a certain cell depends on differentiation due to gene inactivation, and this differentiation is dependent on cellular environment and cell-to-cell contacts. Cellular organisation into specific tissues and organs results in morphologically identifiable structures with known functional capacities.

In disease, normal morphological and functional correlations are often defective. Tumours become dedifferentiated and genes become derepressed. Morphological appearances are abnormal and cellular functions are altered due to loss of some specialised properties, and reactivation of others. Adrenocortical tumours produce abnormal steroid products with varying manifestations. Paradoxically, the more abnormal the process of steroidogenesis, the longer the delay in diagnosis because of the prolonged period of latent growth without systemic upsets. Germ cell tumours often regain the capacity to produce foetal or trophoblastic compounds and these compounds may give the tumour an advantage over the host cells resulting in tumour progression at the expense of normal tissue.

Function in relation to cellular contacts and cell-to-cell integration can be examined in tissue culture. In this respect, monolayer culture is the most exacting and informative system in general use. However, histological relationships are lost in monolayer culture, and morphological examination is confined to a cytological level as was seen in the monolayer cultures of the foetal testis.

When histological relationships *in vitro* are important, organ culture techniques are used. Monolayer culture and organ culture were both used successfully in the experiments of this thesis, and in both cases functional studies were discussed in relation to possible physiological correlations.

Morphology and function must always be studied in relation to each other. Using this approach continuing studies of biological phenomena will lead to a better future understanding of natural processes in health and disease.

SECTION III : GENERAL DISCUSSION AND SUMMARY

CHAPTER 9

SUMMARY

CHAPTER 9

SUMMARY

This thesis deals with experimental aspects of morphological and functional correlations at the histological and biochemical levels in various normal and neoplastic tissues. A physiological section examines functions and effects of the normal foetal testis, and a clinicopathological section is concerned with morphological and functional features of testicular and adrenocortical neoplasms.

The normal foetal testis.

The foetal testis is hormonally active, producing androgenic steroids which cause masculinisation of the sex ducts and urogenital sinus: and a separate Mullerian inhibitory factor (MIF) which actively suppresses the female sex duct. MIF is not a steroid molecule in free form, and at least part of it is a protein. I set up an organ culture bioassay system to study aspects of Mullerian duct inhibition.

Foetal rat genital tracts, containing Mullerian and Wolffian ducts, were explanted from foetal rats and maintained in organ culture for 3 days. The development of gonads and sex ducts *in vitro* followed the normal *in vivo* pattern. The Wolffian ducts were stabilised by androgens, by autologous or homologous foetal rat testes, and by heterologous human foetal testes. The Mullerian ducts regressed in the presence of autologous, homologous or heterologous foetal testes.

On theoretical grounds, I wondered if foetal testicular progesterone, probably attached to a carrier protein analogous to the androgen binding protein produced by the adult testicular Sertoli cells, may be involved in Mullerian duct inhibition. Addition of progesterone to the culture medium did cause Mullerian duct regression, but it was difficult to be certain that this was a specific physiological effect rather than a non-specific cytotoxic effect. Progesterone was also found to stabilise the Wolffian duct in organ culture.

Substantiation of the possible physiological role of foetal testicular progesterone necessitated verification that the foetal testis is capable of progesterone production and secretion. This was attempted using monolayer cultures of rat foetal testes maintained with and without trophic hormonal stimulation. The endogenous steroid production and secretion was determined by radioimmunoassay (RIA) for progesterone and testosterone, and by high pressure liquid chromatography (HPLC) which could efficiently separate, identify and quantitate 47 different standard steroid molecules. A testosterone RIA had been established in our laboratory, and I developed a similar assay for progesterone paying particular attention to precision, sensitivity, specificity and choice of method for separating free and bound labelled progesterone. HPLC was used to examine the profile of steroids secreted by the foetal testicular cells in monolayer culture.

Foetal testes secrete testosterone and progesterone in monolayer culture. Testes from 14½ day foetal rats

initially failed to produce significant amounts of steroid hormone *in vitro*, but after cyclic AMP stimulation substantial amounts of progesterone were identified. Testes from older foetal rats initially produced testosterone in greater amounts than progesterone in culture, but after a few days testosterone, then progesterone, output fell to baseline levels. Steroidogenesis was occasionally reactivated by human chorionic gonadotrophin stimulation, but a greater, more consistent reactivation was effected by cyclic AMP, progesterone production being evident before testosterone.

The results indicate that the foetal testis may produce progesterone at a time when it is known to produce the Mullerian inhibitory factor. Later in gestation, secretion of MIF diminishes, and output of androgens increases. I postulate that this may be due to conversion of progesterone to testosterone. Definite proof of this hypothesis is still lacking.

I conclude from the section on experiments with normal animal tissues that the histological features of foetal testes give no indication of the pattern of steroidogenesis, and that the cytological appearances of foetal testicular cells in monolayer culture do not alter with changing functional activity. The histological regression of Mullerian ducts in organ culture is morphological evidence of the presence of MIF, but it is not truly specific.

The remainder of this thesis concerns neoplastic tissue with secretory potential, and the morphological

patterns associated with various secretory products.

Testicular tumours.

I examined the clinical and histological features of 322 male patients with testicular tumours and extra-gonadal germ cell tumours, and in each case, the serum level of alpha-fetoprotein (AFP) was measured. Many teratomas contain extra-embryonic elements although their significance and identification are not always stressed in authoritative classifications of testicular tumours. About two thirds of teratomas contain extra-embryonic yolk sac elements, and a similar proportion are associated with elevated serum AFP. I found a strong correlation between the presence of yolk sac elements and AFP production, but many exceptions occurred. I suggest, therefore, that the presence of one should arouse the suspicion of the other, but that an absolute and exclusive correlation does not exist.

Yolk sac elements occur in teratomas in patients of all age groups. Pure yolk sac tumours carry a poor prognosis, marginally worse than malignant teratoma undifferentiated (MTU). The presence of yolk sac elements does not adversely affect the prognosis of MTU, but malignant teratoma intermediate (MTI) has a poorer prognosis if yolk sac elements are present. MTI, however, even when yolk sac elements are present, carries a better prognosis than MTU.

Serum levels of AFP may indicate tumour activity in teratoma patients. A persistently elevated serum AFP always indicated active teratoma, and was often a more sensitive index of tumour recurrence than full clinical examination.

However many false negatives occurred and serology alone is insufficient in the monitoring of teratoma patients. Many tumour index substances may be produced by teratomas, but the two most sensitive and reliable markers are the serum levels of AFP, and the beta subunit of HCG. Both should be measured serially in all patients with testicular tumours, and a preoperative sample is very important.

The data presented in this thesis suggest that evidence of AFP production by teratomas may be a poor prognostic sign, but the effect is, at best, marginal. Seminomas carry a better prognosis that teratomas, and the most important prognostic feature of teratomas is the clinical stage at presentation. The presence of extranodal metastases has dire significance.

Adrenocortical tumours.

Adrenocortical tumours have features in common with normal foetal testes, and with testicular neoplasms. They are said to be hormonally active when they produce endocrine imbalance; however some are not associated with endocrine upset. Such tumours are not truly "non-functional". They do not secrete excess active hormones, but are often associated with production of hormonally inactive steroids detectable in the serum or urine. Twenty such "non-hormonal" adrenocortical tumours are presented in this thesis. They are usually large at presentation because of their lack of systemic upset and of their inaccessible position. Pain and abdominal mass are the most frequent presenting features. They are usually malignant and prognosis is poor because of

the prolonged latent period during which time local infiltration and distant metastasis are common.

The histological features of these tumours were examined in order to determine if there are any features associated with degree of malignancy, or steroidogenic potential. As with many other endocrine tumours, the usual criteria for histological grade of malignancy are poorly correlated with clinical behaviour of adrenocortical tumours. The most important pathological prognostic feature is size of tumour at presentation, the larger tumours having a shorter survival. They are histologically very similar to hormonally active adrenocortical carcinomas, therefore the secretory potential cannot be surmised on histological examination alone, and additional functional studies are required. The most important morphological index of tumour function is the appearance of the attached adrenal cortex. A non-hormonal tumour is not associated with trophic changes in the uninvolved gland which may, therefore, be distorted because of tumour bulk, but the normal elements are present in normal proportions. A hormonally active adrenocortical tumour producing active glucocorticoids will, by dint of its negative feedback, result in atrophy of the inner zones of the adjacent (and contralateral) adrenal cortex.

The morphologist has an essential role to play in the examination of normal and pathological tissue, but morphology alone is insufficient. The conclusion of this thesis is that the morphologist and the experimentalist must work together in order to achieve a functional pathological interpretation of disease.

APPENDICES

APPENDIX I

STEROID NOMENCLATURE

Trivial name	Abbreviation	Chemical name
adrenosterone	G	androst-4-ene-3, 11, 17-trione
aldosterone	ALDO	11β, 21-dihydroxy-18-al-pregn-4-ene-3, 20-dione
3α-androstanediol		5α-androstane-3α, 17β-diol
3β-androstanediol		5α-androstane-3β, 17β-diol
androstanedione		5α-androstan-3, 16-dione
androstenediol		5-androsten-3β, 17β-diol
androstenedione	AD	4-androsten-3, 17-dione
androsterone		5α-androstan-3α-ol-17-one
cholesterol	CHOL	5-cholesten-3β-ol
corticosterone	B	4-pregnen-11β, 21-diol-3, 20-dione
cortisol (hydrocortisone)	F	4-pregnen-11β, 17α, 21-triol-3, 20-dione
cortisone	E	4-pregnen-17α, 21-diol-3, 11, 20-trione
cyproterone acetate		1, 2α-methylen-6-chlor-$\Delta^{4,6}$-pregnadien-17α-ol-3, 20-dione acetate
cyanoketone		2α-cyano-4, 4, 17α-trimethyl-androst-5-ene-17β-ol-3-one
11-dehydroaldosterone	DHALDO	21-hydroxy-18-al-pregn-4-ene-3, 11, 20-trione
11-dehydrocorticosterone	A	4-pregnen-21-ol-3, 11, 20-trione
dehydroepiandrosterone	DHA	5-androsten-3β-ol-17-one
11-de(s)oxycorticosterone	DOC	4-pregn-21-ol-3, 20-dione
11-de(s)oxycorticosterone acetate	DOCA	4-pregn-21-ol-3, 20-dione acetate
11-de(s)oxycortisol	S	4-pregnen-17α, 21-diol-3, 20-dione
21-de(s)oxycortisol	21-deoxy F	4-pregnen-11β, 17α-diol-3, 20-dione

Trivial name	Abbreviation	Chemical name
dexamethasone		1, 4-pregnadien-9-fluoro-16α-methyl-11β, 17α, 21-triol-3, 20-dione
20α-dihydroprogesterone	DHP	20α-hydroxypregn-4-en-3-one
20β-dihydroprogesterone		20β-hydroxypregn-4-en-3-one
5α-dihydrotestosterone (androstanolone) (dihydrotestosterone)	DHT	5α-androstan-17β-ol-3-one
5β-dihydrotestosterone (aetiocholanolone)		5β-androstan-17β-ol-3-one
17 ethinyltestosterone		4-androsten-17α-ethinyl-17β-ol-3-one
11β-hydroxyandrostene-dione	11βOH-AD	11β-hydroxyandrost-4-ene-3, 17-dione
19-hydroxyandrostene-dione		19-hydroxyandrost-4-ene-3, 17-dione
18-hydroxycorticosterone	18OH-B	11β, 18, 21-trihydroxypregn-4-ene-3, 20-dione
6β-hydroxycortisol	6βOH-F	4-pregn-6β, 11β, 17α, 21-tetrol-3, 20-dione
18-hydroxy-11-dehydro-corticosterone	18OH-A	18, 21-dihydroxypregn-4-ene-3, 11, 20-trione
18-hydroxy-11-deoxy-corticosterone	18OH-DOC	4-pregnen-18, 21-diol-3, 20-one
11β-hydroxy-20α-dihydro progesterone		11β, 20α-dihydropregn-4-en-3-one
17α-hydroxy-20α-dihydroprogesterone	17αOH-DHP	17α, 20α-dihydroxypregn-4-en-3-one
17α-hydroxy-20β-dihydroprogesterone		17α, 20β-dihydroxypregn-4-en-3-one
17α-hydroxypregnenolone	17αOH-preg	5-pregnen-3β, 17α-diol-20-one
6α-hydroxyprogesterone		4-pregnen-6α-ol-3, 20-dione
6β-hydroxyprogesterone		4-pregnen-6β-ol-3, 20-dione
11β-hydroxyprogesterone		4-pregnen-11β-ol-3, 20-dione
16α-hydroxyprogesterone	16αOH-P	4-pregnen-16α-ol-3, 20-dione
17α-hydroxyprogesterone	17αOH-P	4-pregnen-17α-ol-3, 20-dione
7α-hydroxytestosterone	7αOH-T	4-androsten-7α, 17β-diol-3-one

Trivial name	Abbreviation	Chemical name
11β-hydroxytestosterone		4-androsten-11β, 17β-diol-3-one
16α-hydroxytestosterone		4-androsten-16α, 17β-diol-3-one
19-hydroxytestosterone		4-androsten-17β, 19-diol-3-one
17-isoaldosterone		11β, 21-dihydroxy-18-al-(17β)-pregn-4-ene-3, 20-dione
11-ketoprogesterone		4-pregnen-3, 11, 20-trione
17 methyltestosterone		1, 4-androstadien-17α-methyl-17β-ol-3-one
17α-oestradiol		1, 3, 5(10)-oestratrien-3, 17α-diol
17β-oestradiol	E_2	1, 3, 5(10)-oestratrien-3, 17β-diol
oestriol	E_3	1, 3, 5(10)-oestratrien-3, 16α, 17β-triol
oestrone		1, 3, 5(10)-oestrien-3-ol-17-one
prednisolone (1-dehydrocortisol)		1, 4-pregnadien-11β, 17α, 21-triol-3, 20-dione
prednisone (1-dehydrocortisone)		1, 4-pregnadien-17α, 21-diol-3, 11, 20-trione
pregnanediol		5β-pregnan-3α, 20α-dione
5α-pregnanedione		5α-pregnan-3, 20-dione
5β-pregnanedione (pregnanedione)		5β-pregnan-3, 20-dione
5α-pregnanolone		5α-pregnan-3α-ol-20-one
5β-pregnanolone (pregnanolone)		5β-pregnan-3β-ol-20-one
pregnenolone	Preg	5-pregnen-3β-ol-20-one
progesterone	P	4-pregnen-3, 20-dione
testosterone	T	4-androsten-17β-ol-3-one

APPENDIX II

STEROIDOGENIC ENZYME NOMENCLATURE

Abbreviated name	Recommended name	Systematic name
3β-HSD-I	Δ^5-3β-hydroxysteroid dehydrogenase-isomerase system 3β-hydroxysteroid dehydrogenase and 5-ene-3-ketosteroid isomerase	3β-hydroxysteroid:NAD^+ oxidoreductase (E.C. 1.1.1.51) 3-ketosteroid-Δ^4-Δ^5-isomerase (E.C. 5.3.3.1)
20 : 22 D	20- and 22- hydroxylase desmolase system	no systematic name yet recommended
17α H	steroid 17α-hydroxylase or steroid 17α mono-oxygenase	steroid, hydrogen donor: oxygen oxidoreductase (17α-hydroxylating) (E.C. 1.14.99.9)
17 D	C^{17-20} desmolase	no systematic name yet recommended
17β HSD	17β-oxidoreductase or 17β-hydroxysteroid dehydrogenase or testosterone 17β-dehydrogenase	17β-hydroxysteroid:NAD^+ 17-oxidoreductase (E.C. 1.1.1.63)

Based on Enzyme Nomenclature : Recommendations (1972) of the Commission on Biochemical Nomenclature. IUPAC and IUB. Elsevier : Amsterdam (1973)

APPENDIX III

LABORATORY TECHNIQUES

(a) Glass redistilled water was used for the preparation of all aqueous solutions.

(b) Laboratory glassware

All glassware (except scintillation vials) was soaked in detergent (2% RBS 25 in distilled water), rinsed several times in glass distilled water and finally glass redistilled water before drying in a hot oven.

Radioimmunoassays were performed in new 75 X 10mm glass assay tubes washed as above and discarded after using once.

APPENDIX 1V

RADIOIMMUNOASSAY OF PROGESTERONE

The method is similar to that of Cameron & Scarisbrick (1973).

1. Reagents

Oxygen free nitrogen: British Oxygen Company.

Petroleum ether (boiling point 40°-60°C): BDH "analar" grade.

Ethanol: James Borroughs Ltd. analytical reagent.

Gelatin: Standard Laboratory reagent.

Dextran T-70: Pharmacia.

Washed activated Charcoal: "Norit-A", Sigma. Several grams agitated for 15 minutes in a 1 litre beaker containing approximately 800ml glass redistilled water. Allowed to settle for 5 minutes and supernatant decanted.
This procedure repeated 6 times to remove large (floating) charcoal particles. Residue dried in 140°C oven.

Tritiated progesterone: (1,2,6,7(n)-^{3}H) progesterone, Radiochemical Centre, Amersham, UK. Specific activity 94 curies/mMole. 298 mCi/mg suppied in benzene (1mCi/ml). 250µl of this diluted to 12.5ml in nanograde benzene (Mallinckrodt) to give a stock solution of 20µCi/ml.

Progesterone: Sigma. Recrystallised before use.

Scintillation fluid: Instagel, Packard.

2. Working solutions

Stock phosphate solutions (BDH: Analar):

i) 0.5M NaH_2PO_4: 7.80g of NaH_2PO_4 (MW 156.01) made up to 100ml and stored at 4°C.

ii) 0.5M Na_2HPO_4: 35.82g of $Na_2HPO_4.12H_2O$ (MW 358.16) made up to 200ml and stored at 4°C.

Buffer solutions:

i) 0.9M phosphate buffered saline with azide pH 7.0 ("PBS"):-

7.78g NaCl (BDH Analar)

0.89g NaN_3 (BDH reagent grade)

6.67ml of 0.5M NaH_2PO_4

13.33ml of 0.5M Na_2HPO_4

Made up to approximately 800ml and pH adjusted to 7.0 with NaH_2PO_4 or Na_2HPO_4 stock solutions.

Made up to 1 litre and stored at 4°C.

ii) PBS 0.5% gelatin solution:-

1.25g gelatin dissolved in approximately 200ml PBS using heated magnetic stirrer, and made up to 250ml with PBS to give 5g gelatin per litre of phosphate buffered saline with azide. Stored at 4°C.

iii) PBS 0.1% gelatin solution ("assay buffer"):-

250mg gelatin dissolved in approximately 200ml of PBS using heated magnetic stirrer and made up to 250ml to give 1g gelatin per litre of phosphate buffered saline azide. Stored at 4°C.

Dextran coated charcoal:-

500ml PBS

500mg gelatin

dissolve with heated magnetic stirrer then cool to 4°C

125mg Dextran T-70

Dissolve with magnetic stirrer at 4°C

1.25g washed charcoal

Suspend with magentic stirrer at 4°C for at least 30 min.

Final concentration	gelatin	1g/l
	Dextran T-70	250mg/l
	Charcoal	2.5g/l

Store at 4°C. Agitate for at least 15 minutes at 4°C with magnetic stirrer before use, and agitate with magnetic stirrer at constant speed throughout period of use.

Polyethylene glycol (PEG) 19.5% (w/v):-

39g of PEG made up to 200g with PBS

1 ml of 19.5% PEG added to 0.3ml reaction mixture gives final PEG concentration of 15% (w/v).

Standard progesterone solutions:-

15mg recrystallised progesterone made up to 10ml with ethanol to give 1.5mg/ml stock progesterone solution.

Stored at -20°C.

Stock progesterone solution diluted with ethanol in glass scintillation vials to give 10ml of each of 5ng/ml, 4ng/ml, 3ng/ml, 2ng/ml, 1ng/ml (500, 400, 300, 200, 100 pg/100µl respectively).

Standard progesterone solutions used for standard curves.

Progesterone antiserum:-

0.5ml of 1-in-5 antiserum thawed and diluted to 10ml with assay buffer to give stock 1:100 antiserum.
Stored at 4°C.
Diluted to appropriate concentration (1:2000 to 1:6000) with assay buffer and stored at 4°C for up to 1 week.

Tritiated progesterone label:-

100µl of stock 20µCi/ml in benzene evaporated to dryness under nitrogen at 40°C so that 100µl gives approximately 22,000 distintegrations per minute (DPM).

3. Procedure

i) Pipette in duplicate or triplicate 100µl of progesterone solution (standard, or unknown extract) in analytical grade ethanol at 4°C into cleaned assay tubes (75 X 10mm). 100µl of ethanol is used for zero ("00") on the standard curve; for reagent blank ("RBl"); and for total counts added ("tot").

ii) Dry all tubes at 40°C under a jet of nitrogen

iii) Chill tubes to 4°C and add 100µl of antiserum diluted in assay buffer. 100µl assay buffer used instead of antiserum in "tot". Mix tubes on a vortex and allow to stand for 20 mins at 4°C.

iv) Add 100µl tritiated progesterone in assay buffer (approximately 22,000 DPM). 100µl assay buffer used instead of progesterone label in "RBl". Mix on vortex an incubate overnight at 4°C.

v) Add 100µl PBS with 0.5% gelatin at 4°C. Mix on vortex.

vi) Add 1 ml of dextran coated charcoal suspended in assay buffer at 4°C.

1 ml of assay buffer used instead of charcoal suspension in "tot". Mix on vortex and allow to stand for 5 minutes only. (The dextran charcoal suspension is agitated by a constant velocity Teflon coated magnetic stirrer at 4°C for 2 minutes before use and throughout the time it is being pipetted into the assay tubes).

vii) Centrifuge at 4°C for 12½ minutes at 2400rpm in a Minstral 6L centrifuge.

viii) Decant the supernatant containing the bound progesterone into scintillation vials, add 10ml Instagel and determine the radioactivity (in DPM) in a scintillation counter.

4. <u>Separation of free and bound progesterone: comparison of dextran coated charcoal and polyethylene glycol (PEG)</u>

In step (vi) of procedure, 1 ml of 19.5% PEG is added to the reaction mixture in place of dextran charcoal. This precipitates the bound progesterone leaving free progesterone in solution as opposed to charcoal separation which precipitates free progesterone. The PEG supernatant is decanted into scintillation vials, 10ml Instagel added and the radioactivity determined.

5. Progesterone RIA: Antibody dilution curve

In step (i) of the procedure add 100µl of standard progesterone solution (5ng/ml ethanol) to half of the assay tubes and 100µl ethanol to the others.

In step (iii) add 100µl of varying dilutions of antiserum in duplicate or triplicate to the tubes containing progesterone, and to those containing none.

In step (vi) use dextran coated charcoal or PEG to separate free and bound progesterone.

6. Progesterone RIA: standard curve

In step (i) of procedure pipette in triplicate 100µl of each of the standard progesterone solutions in ethanol (5,4,3,2,1 ng/ml giving 500,400,300,200,100 pg of progesterone in 100µl respectively) into cleaned assay tubes. Use 100µl of ethanol in place of progesterone solution in "OO", "tot", and "RBl".

APPENDIX V

RADIOIMMUNOASSAY OF TESTOSTERONE

The method is similar to that of Collins et al. (1972).

1. Reagents

Testosterone: Sigma. Recrystallised before use.

Toluene: Mallinckrodt. Nanograde reagent.

Gelatin: Standard laboratory reagent.

Dextran T-70: Pharmacia.

Washed activated charcoal: "Norit-A" Sigma.

Washed as for progesterone RIA.

Hydrochloric acid: BDH Analar concentrated HCl.

Tritiated testosterone: (1,2,6,7-^{3}H) testosterone, Radiochemical Centre, Amersham, UK. Specific activity 100 Ci/mMole supplied in benzene. This was evaporated under a jet of oxygen-free nitrogen and taken up in analytical grade ethanol (Borroughs) to give a final concentration of 100uCi/ml. Stored at 4^{o} C. 200ul of this dried under nitrogen and taken up in 2ml of nanograde toluene to give 10µCi/ml.

2. Working solutions

Tritiated testosterone label: 100µl of 10µCi/ml testosterone in toluene dried under nitrogen and taken up in 10ml of Tris assay buffer (vide infra). 100µl of tritiated testosterone in buffer contains approximately 22,000 DPM.

0.1M Tris buffer pH 8.5:-

12.1g Tris-(Hydroxymethyl)-methylamine ("tris buffer" SLR, Fisons).

Dissolved in approximately 850 ml redistilled water.

pH adjusted to 8.5 with approximately 2ml conc. HCl.

Made up to 1 litre with redistilled water.

0.1M Tris buffer pH 8.5 with 0.5% gelatin solution:

1.25g gelatin

Dissolved in approximately 200ml Tris buffer using heated magnetic stirrer.

Made up to 250ml with Tris buffer

0.1M Tris buffer pH 8.5 with 0.1% gelatin solution ("assay buffer")

50ml Tris buffer 0.5% gelatin

200 ml Tris buffer

Dextran coated charcoal:

500ml 0.1M Tris buffer

500mg gelatin. Dissolved using heated magnetic stirrer

125mg dextran T-70. Dissolved using magnetic stirrer at $4^{o}C$.

1.25g washed charcoal. Suspend with magnetic stirrer for 1hour at $4^{o}C$.

Store at $4^{o}C$.

Standard testosterone solution (stock):

24.2mg testosterone made up to 10ml in toluene to give 2.42mg/ml.

Working standard testosterone solutions:

Stock testosterone solution diluted with toluene to give 2.5, 2.0, 1.5, 1.0, 0.5 mg/ml equivalent to 250,200, 150, 100, 50 pg testosterone in 100µl standard solution.

Testosterone antiserum:-

Diluted in assay buffer to a final concentration of 1:4000 to 1:7000.

3. Procedure

i) Pipette 100µl testosterone solution (standard or unknown) in toluene at 4°C into cleaned assay tubes (75 X 10mm) in duplicate or triplicate. 100µl of toluene is used for zero ("00") on the standard curve, for the reagent blank ("RBl") and for the total counts added ("tot").

ii) Dry all tubes at 45°C in a jet of nitrogen

iii) Chill assay tubes to 4°C and add 100µl of anti testosterone serum diluted in assay buffer. 100µl of assay buffer used instead of serum in "tot".

iv) Add 100µl tritiated testosterone in assay buffer (approximately 22,000 DPM). 100µl assay buffer used instead of testosterone label in "RBl". Mix on vortex and incubate overnight at 4°C.

v) Add 100µl Tris buffer 0.5% gelatin at 4°C to all tubes. Mix on vortex.

vi) Add 1ml of dextran coated charcoal suspension in assay buffer at 4°C. 1ml of assay buffer used instead of charcoal suspension in "tot". Mix on vortex and allow to stand for 20 mins at 4°C. (The dextran charcoal suspension is constantly agitated by a Teflon coated magnetic stirrer at 4°C for 20 minutes before use and throughout the period of pipetting into the assay tubes).

vii) Centrifuge at 4°C for 12½ minutes at 2400 rpm in a Minstral 6L centrifuge.

viii) Decant supernatant containing the bound testosterone into scintillation vials, add 10ml Instagel (Packard) and determine radioactivity in DPM in a scintillation counter.

APPENDIX VI

EXTRACTION OF TISSUE CULTURE MEDIA FOR RIA

Five ml of tissue culture fluid was collected daily from each monolayer culture and stored at -20°C till required for extraction when it was thawed at room temperature and 1 ml pipetted into each of two Sovirel glass extraction tubes fitted with Teflon lined plastic screw caps. The tubes were chilled to 4°C. One tube was used for progesterone and the other for testosterone determination.

Procedure:

i a) Progesterone: Add 100µl of tritiated progesterone in PBS assay buffer (approx 22,000 DPM) as internal recovery standard to one tube containing 1 ml of culture medium. Mix on vortex and allow to stand for 15 minutes at 40°C and one hour at 4°C. The total amount of label added is determined by pipetting 100µl of tritiated progesterone in PBS assay buffer into a scintillation vial with 500µl of ethanol and 10ml of Instagel.

i b) Testosterone: Add 50µl of tritiated testosterone in Tris assay buffer (approx 11,000 DPM) as internal recovery standard to other tube containing culture medium. Mix on vortex and allow to stand for 20 minutes at 4°C. The total amount of label added is determined by pipetting 50µl of tritiated testosterone in Tris assay buffer into a scintillation vial with 400µl of toluene and 10ml of Instagel.

ii a) Progesterone: Add 3ml of petroleum ether (B.P. 40-60°C) at 4°C to extraction tube, firmly stopper and invert manually 120 times in cold room. Allow to settle for 10 minutes.

ii b) Testosterone: Add 2ml of nanograde toluene at 4°C to extraction tube, firmly stopper and invert manually 120 times in cold room. Allow to settle for 10 minutes.

iii a) } Freeze lower aqueous layer by emersing glass tube
iii b) } in ethanol cooled by solid carbon dioxide, and decant upper solvent layer into clean glass scintillation vials.

iv a) } Repeat steps (ii) and (iii) pooling the solvent layers
iv b) } into scintillation vials to dryness at 40°C (petroleum ether) or 45°C (toluene) under a jet of nitrogen.

v a) Progesterone: Dissolve the residue in the scintillation vial with 2.5ml ethanol at 4°C. Transfer 500µl of this to a fresh scintillation vial with 100µl PBS assay buffer and 10ml of Instagel: this is 20% of total label recovered by the extraction procedure.

v b) Testosterone: Dissolve the residue in the scintillation vial with 2.0ml toluene at 4°C. Transfer 400µl of this to a fresh scintillation vial with 50µl Tris assay buffer and 10ml Instagel: this is 20% of the total label recovered by the extraction procedure.

vi a) Progesterone: } <u>Ranging assay</u>: Pipette 20,50,100,
vi b) Testosterone: } 200µl of extracted steroid in solvent to clean assay tubes at 4°C. Evaporate to dryness at 40°C (Progesterone) or 45°C (Testosterone) under nitrogen and determine approximate amount of steroid by RIA.

Precise assay: Dilute extracted steroid with ethanol (Progesterone) or toluene (Testosterone) to give a final concentration of approximately 2ng/ml (Progesterone) or 1ng/ml (Testosterone). Determine amount of steroid in 100µl of diluted extract by RIA and calculate total amount of steroid in 5ml of culture medium.

APPENDIX VII

PREPARATION OF TISSUE CULTURE MEDIA

A. CULTURE MEDIA FOR ORGAN CULTURE OF FOETAL GENITAL TRACTS

1. Preparation of solid (agar) medium

8ml of medium 199 (X 2 concentration) [Flow laboratories]
with Hank's balanced salt solution
with 40mM HEPES buffer
with glutamine
without bicarbonate
without phenol red

4ml of foetal calf serum (Gibco)

100µl of Penicillin (5000 units/ml)
Streptomycin (5000 units/ml)
Neomycin (10 mg/ml) [Gibco]

200µl of 5.6% Na bicarbonate

1 drop phenol red

→ MEDIUM A

6ml of medium A
4ml of glass redistilled water

→ MEDIUM B (See Table A.I)

6ml of medium A at 37°
4ml of sterile 2% aqueous agar at 37°C

→ MEDIUM C (See Table A.I)

Table A.I. Concentrations of constituents of tissue culture media used for organ culture of foetal rat genital tracts on solid media.

Constituent	Medium B	Medium C
Medium 199 (with Hank's salts)	X 1	X 1
HEPES buffer	20 mM	20 mM
Agar	0	0.8 %
Bicarbonate	0.56 g/l	0.56 g/l
Foetal calf serum	20 %	20 %
Penicillin / streptomycin	25 units/ml	25 units/ml
Neomycin	50 μg/ml	50 μg/ml
Amphotericin	1.25 μg/ml	1.25 μg/ml

The 2% aqueous solution of agar is sterilised by autoclaving at 15 lbs/sq. in. for 20 minutes and allowed to cool to 37°C at which time 4ml is added to 6ml of medium A, immediately mixed, and 1 ml aliquots pipetted into special moulds placed in sterile Petri dishes. When medium C cools to room temperature it forms a solid gel and the special moulds are carefully removed leaving a rectangular ridge of solid culture medium measuring 30 x 7 x 4mm in the Petri dish.
The final concentration of the constituents of media B & C are shown in Table A.I.

2. Preparation of liquid medium

16 ml of medium 199 (X 1 concentration) [Flow laboratories]
with Hank's balanced salt solution
with 20mM HEPES buffer
with glutamine
with phenol red
with bicarbonate
4ml foetal calf serum [Gibco]
100µl of Penicillin (5000 units/ml)
Streptomycin (5000 units/ml)
Neomycin (10 mg/ml) [Gibco]
200µl of Amphotericin (250mg/ml) [Gibco]
→ MEDIUM D

Final concentration of constituents of medium D is similar to that of medium B.

B. CULTURE MEDIA FOR MONOLAYER CULTURE OF FOETAL TESTES

1. "Dissecting medium"

100ml of medium 199 [Flow laboratories]
with Earles' salts
with 20mM HEPES buffer
with glutamine
without bicarbonate
2ml of Penicillin (5000 units/ml)
Streptomycin (5000 units/ml) [Gibco]
2ml of Kanamycin (10mg/ml) [Gibco]
400µl of Amphotericin (250mg/ml) [Gibco]
16.7ml of Foetal calf serum (virus and mycoplasma screened) [Gibco]

2. "Enzyme solution"

12.5mg collagenase (Sigma)
12.5mg hyaluromidase (Sigma)
Stored dry at -20°C in sterile plastic universal container.
Before use, 5ml of "dissecting medium" is added to give a final concentration of 2.5mg/ml of each enzyme.

3. Dialysed foetal calf serum

The monolayer cultures were maintained in a glutamine restricted environment to minimise cell proliferation, therefore the foetal calf serum used in the culture medium was dialysed to remove amino acids, especially glutamine. Dialysis also removes steroid hormones, which could interfere with the steroid assays, and polypeptide hormones which could stimulate the cells in culture.

Virus and mycoplasma screened foetal calf serum (Gibco) was poured into Visking dialysis tubing which was then knotted at each end and placed in a 2 litre beaker full of sterile 0.09% NaCl (Steriflex:BDH). Usually 4 x 50ml of serum was dialysed in each batch. The saline was agitated with a magnetic stirrer. The serum was dialysed for 48 hours, the saline being renewed twice daily.

4. "Culture medium"

100ml MEM (Eagle) [Gibco]
with Earles' salts
without l-glutamine
2ml of 7.5% Na bicarbonate [Gibco]
2ml of Penicillin (5000 units/ml)
Streptomycin (5000 units/ml) [Gibco]
200µl of l-glutamine (100 x concentration) [Gibco]
16ml dialysed foetal calf serum

The medium was depleted of glutamine in order to reduce cell division because the metabolism of non-proliferating cells was under study. However, over-zealous glutamine restriction was found to be toxic to some cultures and subsequently a small amount of l-glutamine was added to maintain cell viability.

When all the ingredients were added, the medium was sterilised by filtration through 0.45µm and 0.22µm (pore size) Millipore or Sartorius filters at 10 lbs/sq.in. pressure supplied by a cylinder of 10% CO_2 in air. The filtered medium was collected in sterile bottles and stored at $4^{o}C$.

APPENDIX VIII

HIGH PRESSURE LIQUID CHROMATOGRAPHY (HPLC)

1. Extraction of tissue culture media for HPLC

Five ml of tissue culture medium was collected each day from the monolayer cultures of foetal rat testes. Each 5ml sample was stored individually at -20°C in polystyrene screw-capped bijoux (Falcon) bottles until ready for extraction.

a) Thaw tissue culture medium at room temperature.

b) Pipette 2-3ml medium into cleaned glass 25ml Sovirel extraction tube at 4°C.

c) Add 10ml of nanograde dichloromethane (Mallinckrodt) at 4°C and tightly replace Teflon coated screw cap.

d) Extract steroids into dichloromethane by manual inversion 100 times at 4°C.

e) Separate aqueous and solvent layers by centrifugation at 4°C for 7½ minutes at 2000 rpm (Minstral 6L centrifuge). Discard upper aqueous layer.

f) Add 3ml of 0.1N NaOH, replace screw cap and invert 100 times at 4°C. Separate aqueous and solvent layer by centrifugation and discard upper aqueous layer. Repeat 0.1N NaOH extraction. This helps to remove saponified lipids which may interfere in the chromatogram.

g) Transfer dichloromethane layer to clean glass scintillation vial and evaporate to dryness at 40°C under a jet of oxygen-free-nitrogen (BOC).

h) Redissolve residue in approximately 0.5ml of dichloromethane and transfer to a bacteriological glass bijoux tube. Evaporate to dryness under N_2 at 40°C and redissolve in 20µl of 50% ethanol.

i) Inject 10µl of ethanolic extract into HPLC and obtain corresponding chromatogram.

Recovery experiments show that 10µl of the ethanolic extract contain 42.5 ± 1.5% of the total steroid in the original 2-3ml of tissue culture medium. Therefore, total recovery of steroids in 20µl of ethanolic extract is 85 ± 3% (O'Hare et al., 1976b).

2. HPLC systems used

For the separation of the steroids produced by the testis in tissue culture, reversed-phase chromatography with gradient elution through a stainless steel precision bore tubing column 25cm long and 2.1mm internal diameter, commercially packed with Zorbax-ODS (Du Pont) was used. "Zorbax" packings consist of totally porous small particles of 5-10µm diameter. ODS (octadecyl hydrocarbon stationary phase) is the stationary non-polar phase chemically bonded to the Zorbax particles and can be used with aqueous or polar mobile phases of pH values between 2 and 9 at temperatures up to 75°C.

Two solvent systems were used for separation of steroids: acetonitrile/water, and dioxane/water. In the acetonitrile system,

the column was equilibrated with 32% (v/v) acetonitrile in glass redistilled water, and the steroid sample in 50% ethanol injected into the column. The solvent strength was increased from 32% to 100% acetonitrile over 50 minutes using a concave exponential gradient with the configuation $y = x^3$. The elution of UV absorbing steroids was detected spectrophotometrically at 240mm, and the retention time was measured from the moment of injection. The column was washed for 10 minutes with 100% acetonitrile to remove non-polar compounds in the media (cholesterol, etc.) and re-equilibrated with 32% acetonitrile for 25 minutes before injection of the next sample. The temperature of the column was 45°C and the inlet pressure was 2000 psi giving an initial flow rate (32% acetonitrile) of 0.38ml/min, and a final flow rate (100% acetonitrile) of 0.80ml/min

Elution with dioxane as mobile phase was similar to acetonitrile. The solvent strength was increased from 20% (V/V) dioxane in glass redistilled water, to 100% dioxane over 50 minutes using the same $y = x^3$ gradient. With the column temperature at 45°C and inlet pressure of 2500psi, the initial flow rate was 0.38ml/min falling to 0.3ml/min at the end of the gradient. Steroids were detected spectrophotometrically by absorbance at 254nm.

Highly purified acetonitrile and dioxane (Nanograde: Mallinckrodt, St. Louis, Mo. USA; or HPLC grade: Rathburn, Walkerburn, Scotland) gave low background noise and 2ng of steroid resulted in detectable elution peaks on the chromatograph. The peak heights were directly proportional to the quantity of steroid injected over a 50-fold range up to 2000ng. Forty-seven standard steroids have been applied to the column

The steroids produced by the testis in tissue culture can be separated adequately by the acetonitrile or dioxane systems, and other less polar steroids have been separated by a methanol/water system so that all 47 steroids can be identified as separate peaks. The retention times for the standard steroids using acetonitrile and dioxane systems are shown in Table 2.II and the chromatograph for a series of 7 standard steroids is shown in Fig. 2.4.

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