OBSERVATIONS ON THE MAMMALIAN TESTIS

by

A.H. Baillie.

Thesis for the degree of D.Sc.

artment of Anatomy,
niversity of Glasgow.

October, 1963.

INTRODUCTION

In man the more obvious effects of castration have been recognised since ancient times. The distribution of body hair, the pitch of the voice, the contours of the skeleton and the extent of the facial sinuses are familiar examples of morphological characteristics which reflect the endocrine function of the testis.

The first experimental demonstration of hormone production by the testis took place in 1794 when Hunter showed that the rudimentary spur of a hen would develop into a well formed masculine spur if transplanted into the leg of an adult cock, while the small spur of an immature cock remained poorly formed if implanted into a hen's leg. Towards the end of the 19th century, following a demonstration of the efficacy of testicular grafts in alleviating the consequences of castration in fowls, numerous investigators busied themselves with the rejuvenating effects of testicular extracts on the senescent human subject. These attempts to discover an elixor of life were superseded at the beginning of this century by a more rational experimental approach to the problem of endocrine function of the testis. Injections of testicular tissue were found to precipitate the development of the skeleton and comb of young capons, while saline extracts of the testis injected into hens

put a stop to egg laying and resulted in the development of comb and wattles along a masculine pattern. Suspensions of pig testis (Burroughs) produced a similar comb and wattle development in castrated cocks. The changes described in hens and castrated cocks were reversed when the injections were discontinued. The interstitial cells of the testis were first described in 1850 by Leydig, who noted masses of fat laden cells in the spaces between the seminiferous tubules. Interest in these cells as a possible site of male sex hormone, or androgen production, dates from the researches of Bouin and Ancel , who in 1903, postulated an endocrine function for what they termed the"glande interstitiel They suggested on the basis of experimental findings that these cells were responsible for the production and release of humoral agents which control the sex behaviour and secondary sex characteristics of the male.

The term androgen is used as a collective title for those compounds which resemble testosterone in biological action. All the known androgens are steroids being derivatives of the parent substance perhydrocyclopentenophenanthrene. At present three principle groups of androgens are recognised, namely the androsterone derivatives, the testosterone derivatives and the diols and diketones. While these compounds differ quantitatively in their biological activity, their functions in the

vertebrate body are similar and specific. Although the effects of androgen on the various somatic tissues are of considerable physiological significance, from the experimental point of view it is important to note that fluctuations in androgen released by the testis are accurately reflected by quantitative changes in androgen controlled characters. Androgen production may thus be assessed by observing alterations in the weight or cytology of a target organ such as the prostate or seminal vesicle, (Hooker, 1942.) A recent correlation has been established in the bull between the testicular content of testosterone and androstenedion and the content of fructose and citric acid in the seminal vesicles, Hay, Lindner and Mann, 1961.

Morphology of the Leydig cell. In the mature mammalian testis Leydig cells occur in groups between the seminiferous tubules and are large polyhedral cells some 14 or 21 microns in diameter. They usual contain a single spherical or ovoid nucleus with one, two or three slightly eccentric nuclei. Most of the remaining chromatin forms an attenuated net lining the nuclear membrane. Associated with the nucleus two spherical or rod-shaped centrioles may be seen within the attraction sphere. In inflammatory lesions of the testis and in tissue culture mitotic figures have been observed. In the fresh condition the cytoplasm contains numerous refractile granules which react positively

to tests for lipids. Some of these granules have a brownish colouration due to contained pigment. In advanced age, some vitamin deficiency states and at the end of the rut in seasonal breeders, this pigment undergoes a marked increase. It takes the form of osmiophilic granules often aggregated around the periphery of homogeneous vacuoles which do not reduce osmium. It is insoluble in acetone and is frequently referred to as lipochrome pigment. In addition to lipid and pigment granules the cytoplasm also contains numerous spherical or rod-shaped mitochondria, a fine perinuclear Golgi apparatus and the crystalloids of Reinke.

The electron microscope has revealed that mature Leydig cells have a remarkably heterogeneous cytoplasm containing in addition to the usual organelles, large numbers of small membrane limiting vesicles, clusters of small granules of appreciable density, droplets of lipid, aggregates of osmiophilic pigment and, in man, conspicuous protein crystals of a highly ordered internal structure. These crystals have a fabric pattern structure, which may be resolved into a series of densities 100 Å or so in diameter, spaced in a uniform distance of 186 Å apart along two axis, which are at approximately right angles to one another. This pattern is thought to be due to the orderly arrangement of protein macromolecules in crystalline

lattice. Details of the third axis are not yet available.

Histochemistry of the Leydig cell. In all vertebrates so far examined the Leydig cell cytoplasm stains pale pink when exposed to the PAS reaction. In poikilotherms glycogen has been reported in the cytoplasm of Leydig cells, but in homiothermal animals the Leydig cells do not appear to contain this carbohydrate. Other peculiar granules have been described by various workers which are perhaps glycoprotein. The distribution of lipids in the intertubular cells of Leydig as demonstrated by the Sudan stain as plasmal reaction, dinitrophenyl hydrazine, nile blue sulphate, birefringence and autofluorescent studies is extremely variable. Different cells of the same testis give positive reactions to a variable number of these tests, while Leydig cells from the testis of different individuals and species exhibit an even greater diversity of lipid characteristics. Cytoplasmic basophilia is a prominent feature of immature human Leydig cells and also of the Leydig cells of rutting animals. Enzymes demonstrated in the Leydig cell include alkaline phosphatase, an acid phosphatase and 3β-hydroxysteroid dehydrogenase. In common with other steroid producing endocrine tissues the interstitial cell of Leydig contains abundant cytoplasmic ascorbic acid.

Life history of the Leydig cell. Until comparatively recently there has been a good deal of dispute regarding the embryonic origin of the interstitial cell. At present, however, the current consensus of opinion is that Leydig cells differentiate from fibroblasts or similar elements lying between the differentiating seminiferous tubules. Alternative embryological origins postulated for the Leydig cells include leukocytes, lymphocytes, plasma cells, capillary endothelial cells, the sex cords, sertoli cells and the salomic epithelium. The morphological changes constituting maturation or metamorphosis to which this Leydig precursory subject are fairly well known. In the first instance the elongated nucleus becomes round or oval and vesicular, the cytoplasm then increases in amount, it loses its processes and it becomes slightly basophilic. Lipids make their appearance and the nuclei become the centres of alkaline phosphatase activity in a few species. This process is completed by the loss of nuclear alkaline phosphatase and cytoplasmic basophilia and the appearance of abundant steroids, lipids and ascorbic acid. Virtually all recent writers accept the existence of two reasonably distinct generations of Leydig cells in most species studied. The first is a fecal generation which develops in uteral and dies out at or

about birth. The second generation is said, Gillman,1948, Hooker,1948, to appear at puberty and to persist throughout adult life. It would appear necessary, in view of the many literary reports of senile and seasonal Leydig cell atrophy, to postulate a fate for the Leydig cell. Some writers suggest that these cells undergo spontaneous autolysis in certain conditions and that reversion to mesenchyme also occurs. It must be recorded, however, that to date no satisfactory quantitative studies have been made of this problem.

Testicular source of androgen. It has taken biologists more than half a century to establish beyond reasonable doubt that the Leydig cell is the site of androgen production in the testis. In the first instance it was observed that experimental procedures such as artificial cryptorchidism, ligation of the vasa efferentia and exposure to ionising radiations, which produce severe tubular damage, fail to produce castration changes. This suggested that the Leydig cells which survive such assaults might be a source of androgen. Against this view, however, has been the failure to date to correlate changes in the secondary sex characteristics with corresponding changes in the testicular Leydig cell calculation. More recently is has been recognised that the gonadotropins only evoke androgen secretion if they produce visible

morphological changes in the Leydig cell. Further artificially induced Leydig cell hyperplasia and neoplasia is associated with a high level of androgen production, Hooker and Pfiefer,1942. The histochemical studies of Pollok,1942, while suggesting that the Leydig cell is the most likely site of steroid metabolism in the testis have failed to rule out the Sertoli elements which satisfy most of the criteria fulfilled by the Leydig cell. It has been established by Howard in 1950 that the sertoli cells in the seminiferous tubules do produce a steroid hormone, namely Δ -5-pregnenolone or the X hormone. This hormone is believe to inhibit pituitary release of the follicle stimulating hormone (FSH). To stimulate pituitary release of the luteinizing hormone(LH) and to promote spermatogenesis. Justification for this statement rests in the observations that the Sertoli cells mature histochemically at puberty, but lesions of the seminiferous tubules damaging the Sertoli cells are invariably associated with a high urinary FSH titre and that sertoli cell tumours in dogs do produce such a humoral agent in demonstrable amounts. Humoral control of the Leydig cell function must of necessity include a consideration of the factors which evoke morphological and functional differentiation of the mature cell from its indifferent mesenchymal precursor, as well

as those which control the synthesis and liberation of androgen.

Two anterior pituitary hormones, FSH and LH, are thought to be implicated in the Leydig cell life cycle. In any given male there is usually a close parallelism between FSH output estimated by urinary excretion and the numbers of Leydig cells. In conditions of reduced hypophyseal FSH release, as may for example follow oestrogen administration, the Leydig cells tend to be rare or absent. Pathological states, such as sclerotic tubular degeneration, characterised by a high FSH urinary titre are frequently associated with an increase in the volume of Leydig tissue. No constant relationship between FSH production and androgen release has been demonstrated. If is therefore thought that FSH is concerned with the differentiation of the Leydig cells rather than with regulation of their function. Most workers, Greep et al,1946, agree that LH administration evokes some Leydig cell hypertrophy and the secretion of androgen. It seems that differentiation of the Leydig cells from their precursors is induced by traces of FSH contaminating the gonadotropins in experimental use, while the raised androgen secretion is solely due to the LH component. Studies of this nature are unfortunately often complicated by the development of antibodies to the gonadotropins in current use. This thesis embodies work

work which commenced in 1957 and set out to answer several specific questions.

Firstly, what are the histological and histochemical characteristics of the fetal Leydig cell? Is it possible in the fetal testis to demonstrate by histochemical means transitions between mesenchymal precursors and typical Leydig cells?

Secondly, in the post-natal animal do Leydig cells arise by differentiation from mesenchymal or by division of pre-existing Leydig cells? In other words, in the post-natal animal can typical Leydig cells undergo mitosis?

Thirdly, is it possible to relate any intracellular inclusion or organelle to androgen production?

Fourthly, is it possible in a non-seasonal breeder, such as the mouse, to induce changes by artificial means such as exposure to cold analagous to those exhibited by cyclical breaders during the regressive phase? If so, can these changes be related to any histological or histochemical events in the Leydig cell?

Fifthly, what is the effect, if any, of high epididymal obstruction on the histochemical characteristics of the Leydig cell?

The remainder of the thesis contains thirteen parts, each one of which is a manuscript of a paper already

published in a Journal or currently in press. It may readily be subdivided into several portions. The first section contains only one paper and deals with the histochemistry of the Leydig cell in the testis of the fetal sheep. The second section contains five papers and deals with various aspects of the growth, differentiation, mitotic potential and histochemistry of the Leydig cell between birth and adult life. Some ultrastructural observations on the growing Leydig cell are included in this section. The third section deals with the differentiation of the basement membrane of the mouse seminiferous tubule and in a way is a by-product of the second section. Histochemical findings are mentioned and details of ultrastructural changes which take place in the growing basement membrane are also recorded. The fourth section contains two papers on the effects of epididymal obstruction on the testis. The first paper commenced as an attempt to investigate the histochemistry of the Leydig cell following epididymal obstruction. As will be seen from the text it was not found possible to confirm the reports of other workers following this sort of experiment and accordingly a much wider investigation had to be undertaken in order to explain the discrepancies between the current work and that of other people. The fifth section

contains one short paper dealing with the histochemistry and ultrastructure of the gonocyte. The sixth and last section contains two papers which deal with the histochemical and biochemical changes in the reproductive tract of the mouse, following exposure to artificial winter conditions with maintained environmental temperatures at $-3^{o}C$.

The Journal which has published or is publishing each part is indicated immediately below the title of the appropriate part.

THE INTERSTITIAL CELL IN THE TESTIS OF THE FOETAL SHEEP.

Quart. J. micr. Sci. (1960), 101, 4, 475-480.

SUMMARY

The testes of 18 foetal sheep, of crown-rump length from 2.8 to 40 cm, have been studied by the PAS, Sudan black, and plasmal tests. Typical interstitial cells differentiate from mesenchymal precursors; the nucleus alters first, becoming ovoid and vesicular; then the cytoplasm increases in amount, its processes become fewer and smaller, sudanophil lipid droplets appear, and the result is a lipid-laden epitheloid cell. Two atypical forms of interstitial cell have been noted: the first has groups of eosinophil granules in its cytoplasm, the second is shrunken and has a pycnotic nucleus. The interstitial cell of foetal sheep, unlike that of poikilotherms, contains no glycogen. The PAS-positive polysaccharide/protein granules found in the interstitial cells of adult hormiotherms are absent from the interstitial cells of this foetal homiotherm. While sudanophil lipids appear at an early stage, Schiff-positive lipids (plasmalogens, acetal phosphatides, and possibly steroids) are entirely absent from the interstitial cells of the foetal sheep.

INTRODUCTION

Until comparatively recently there has been considerable controversy regarding the precise foetal origin of the testicular interstitium; embryonic sources suggested by various writers for the interstitial cell include leucocytes, lymphocytes, plasma cells, capillary endothelial cells, the sex cords, Sertoli cells, and the coelomic epithelium (Gillman, 1948). Now, however, it is widely accepted, in accordance with Gillman (1948), that the interstitial cell develops from small spindle-shaped elements lying between the seminiferous tubules. This intertubular tissue is derived in the first instance from the general mesenchyme of the primordial testis, but it also receives a contribution of mesonephric mesenchyme accompanying the blood-vessels. It thus appears that the interstitial cell arises from a stellate precursor which is morphologically indistinguishable from the cells giving rise to the cellular elements of ordinary connective tissue.

Although much time and attention have been devoted to the histogenesis of the foetal interstitial cell, there is little information available at present regarding its histochemical characteristics. This work is a study of some of the histochemical features of the foetal interstitial cell; it also seeks to furnish histochemical evidence in support of a mesenchymal origin of the interstitial cell.

MATERIAL AND METHODS

In all, 18 male sheep embryos were studied; the crown-rump length varied from 2.8 to 40 cm. The details are tabulated as follows:

Crown-rump length (cm)	No. of embryos	Position of testes
2.8	1	medial to kidney
8.1	1	abdominal
9.7	3	"
17.5	3	inguinal canal
21.3	2	"
24.3	1	"
31.0	2	scrotal
33.0	2	"
36.0	1	"
40.0	2	"

One testis from each animal was fixed intact in a mixture of 90 ml water, 10 ml formalin, and 5 g mercuric chloride, dehydrated in cellosolve, embedded in ester wax, and sectioned at 5 μ. The stains used were haematoxylin and eosin, and also the McManus/Hotchkiss periodic acid/Schiff (PAS) technique (Carleton and Drury, 1957). It has been shown (Vallance-Owen, 1948) that glycogen is as well fixed by formaldehyde fixatives as by alcohol or alcohol/picric mixtures; the former were found to give vastly superior histological results in the testis, shrinkage of interstitial tissue being minimal by this method.

With the exception of the smallest embryo, the

other testis was in each case fixed in formaldehyde-calcium solution (90 ml water, 10 ml formalin, 1 g anhydrous calcium chloride), embedded in gelatin, and sectioned at 10 μ on the freezing microtome. Some frozen sections were coloured in Sudan black to demonstrate total lipids, while others were subjected to Hayes's modification of Feulgen and to Voit's plasmal reaction (Lillie,1954) to show acetal phosphatides and possibly steroids (Dempsey,1948).

RESULTS

To avoid repetition, descriptions of the specimens stained with haematoxylin and eosin are presented conjointly with descriptions of the corresponding PAS slides.

Results with haematoxylin and eosin and with PAS. The gonad of the 2.8 cm embryo is a mesenchymal mass, covered with cuboidal coelomic epithelium, located in the dorso-medial angle of the coelom; it is coextensive with the developing kidney. The primordium consists solely of stellate mesenchymal cells (fig.1,A), whose membranes are well defined by the PAS procedure. Occasional red, refractile, PAS-positive, diastase-resistant granules may be seen in some cells. The nuclei are usually large and oval. A few large hexagonal cells with vesicular nuclei are visible at the periphery of the primordium; these may be the primitive sex cells. No PAS-positive ground

substance is visible.

The gonads of the 8.1-cm and 9.7-cm embryos resemble one another closely. The spaces between the differentiating seminiferous tubules are populated with stellate mesenchymal cells resembling those described in the 2.8-cm embryo; these cells are connected by transition forms with peritubular fibroblasts on the one hand and with interstitial cells on the other. The latter have abundant hexagonal or rounded cytoplasm, which stains pale pink with eosin; there are no PAS-positive cytoplasmic inclusions. Their nuclei are ovoid and vesicular and possess a characteristic chromatin arrangement.

The first visible event in the differentiation of the typical interstitial cell from its mesenchymal precursor is alteration of the nucleus. The nucleus, originally oval and containing dust-like chromatin, becomes vesicular and round, while the chromatin becomes localized just inside the nuclear membrane; one or more nucleoli become visible. Cytoplasmic differentiation is a slower process than nuclear differentiation. Initially the cytoplasm increases in amount, its processes diminish in size and number before finally disappearing, and the cell-body assumes a polygonal or epitheloid appearance. At a variable stage during this phase (see below), sudanophil droplets make their appearance.

The testes of the 17.5-cm, 21.3-cm, and 24-cm embryos present a uniform histological appearance. There are three recognizable types of interstitial cell:

(1) the most common form has abundant cytoplasm, which colours pink with eosin; the cell-membrane stains clearly with PAS (fig. 1, B); the nucleus is vesicular and ovoid.

(2) the second form differs from the first in that it has variable numbers of prominent eosinophil granules in its cytoplasm (fig. 1, C). The granules occur in groups and may fill a cell process or even the whole cell-body: they are not PAS-positive.

(3) the third form has scanty eosinophil cytoplasm and a shrunken pycnotic nucleus. The cytoplasm is PAS-negative.

The testicular histology of the last four embryo sizes is similar: central differentiation of the tubules and intertubular tissue is beginning to catch up on peripheral differentiation. Only typical interstitial cells persist; the pycnotic forms and those with eosinophil granules have largely disappeared. As in earlier testes, staining with haematoxylin and eosin or with PAS reveals transition forms between stellate mesenchymal cells and typical interstitial cells.

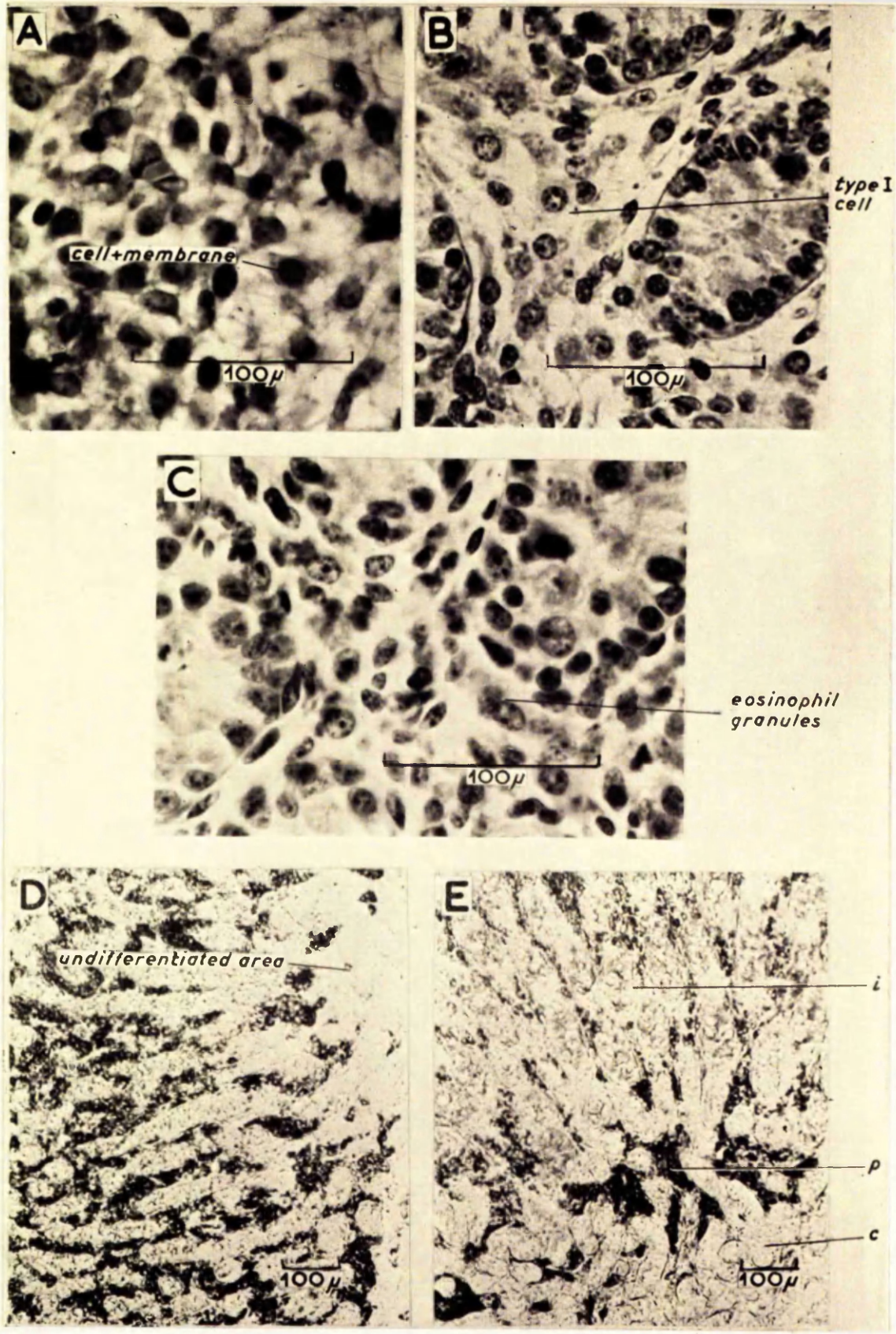


FIG. 1 (plate). A, testis of 2·8-cm embryo. 5 μ, PAS. The gonad consists entirely of stellate mesenchymal cells.

B, testis of 24-cm embryo. 5 μ, PAS. Note the absence of Schiff-positive material in the cytoplasm of the interstitial cells.

C, testis of 17·5-cm embryo. 5 μ, haematoxylin and cosin. Some epithelioid interstitial cells have eosinophil cytoplasmic granules.

D, Testis of 9·7-cm embryo. 10 μ, Sudan black. The centre of the gonad is poorly differentiated: the radial arrangement of the tubules in the remainder is conspicuous.

E, testis of 24-cm embryo. 10 μ, Sudan black. Note the lack of interstitial lipids in the centre (*c*) of the gonad, the intense sudanophilia of the paracentral (*p*) interstitial cells, and the radial arrangement of interstitial cells in the intermediate (*i*) zone.

Results with Sudan black. The testes of the 8.1-cm and 9.7-cm embryos are alike (fig. 1,D). There are no sudanophil elements in the centre of the testis, where differentiation of the various constituents is incomplete. Elsewhere sudanophil interstitial cells are arranged radially in columns of 2 to 8 cells between the seminiferous tubules. The abundant cytoplasm of the individual cells contains numerous minute sudanophil particles: the ground cytoplasm and nuclei are not coloured. Nondescript cells, intermediate in size and shape between typical interstitial cells and mesenchymal cells, are also visible: their cytoplasm contains a variable number of sudanophil inclusions.

The testes of the 17.5-cm, 21.3-cm, 24-cm, 31-cm, and 33-cm embryos resemble one another. The tubules are well defined in the centre of the organ and the interstitial cells may be divided into 4 groups or zones. In the first or peripheral zone, which lies just below the tunica albuginea, the cells are arranged in clumps and have sudanophil cytoplasmic granules. The cells of the second or intermediate zone (fig. 1 E) are histologically similar but are arranged in radial columns. In the third or paracentral zone, which circumscribes the area of newly-defined seminiferous tubules, the cells are coloured very deeply indeed by Sudan black and their cytoplasm is packed

with dust-like black particles. In the centre of the testis the last or fourth group of interstitial cells is imperfectly differentiated; these 'immature' cells have no sudanophil cytoplasmic inclusions. At this stage, however, it will be noted that 'typical' interstitial cells are visible in the centre of the testis in the corresponding material stained with haematoxylin and eosin.

The testes of the 36-cm and 40-cm embryos only differ from the above description in so far as the cells of the central zone have acquired sudanophil inclusions and are not distinguishable from those of the paracentral zone.

Results with the plasmal test. At no time do the interstitial cells contain Schiff-positive lipids (plasmalogens, acetal phosphatides, and possibly steroids); nor is there any evidence of plasmalogens in the Sertoli cells.

DISCUSSION

Most recent workers agree with Gillman's (1948) statement that the testicular interstitial cell arises from a spindle-shaped mesenchymal precursor. In the foetal sheep testis, while many of the peripheral interstitial cells make their appearance during a period not covered by the present available material (i.e. between the 2.8-cm and

the 8.1-cm stage), the more centrally placed cells can be seen to arise by transformation of mesenchymal elements derived from the undifferentiated central portion of the gonad. A similar, centrally placed portion which differentiates more slowly than the remainder of the gonad has been described in the foetal rat testis (Roosen-Runge and Anderson, 1959). On the basis of the PAS and Sudan tests, one can easily recognize that histochemical and histological metamorphosis of the interstitial cell from a mesenchymal precursor does occur.

In the intertubular spaces of testes traversing the inguinal canal (crown-rump length 17.5 cm, 21.3 cm, and 24 cm), there are three types of interstitial cell. While the occurrence of the typical hexagonal epithelioid cell is to be expected, the significance of the shrunken pycnotic forms and of the clumps of eosinophil granules in otherwise cytologically typical cells is problematical. Abnormal shrunken cells are also to be seen in the postnatal mouse testis; they may represent atrophic or regressive forms of interstitial cell.

In man and the horse (Gillman, 1948) foetal interstitial cells reach a developmental maximum at or about mid-term. Thereafter the interstitial tissue undergoes a reduction whose occurrence and timing have not been satisfactorily explained. By contrast, in the rat

(Roosen-Runge and others,1959), in the rabbit (Allen,1903), and in cattle (Bascom,1923) similar interstitial cell maxima do not occur until birth. The ensuing interstitial tissue regression in these animals has been ascribed to deprivation of maternal oestrogens and gonadotrophins. In the present material there is no histological or histochemical evidence, at any stage, of significant interstitial cell degeneration; the post-natal fate of these cells in the sheep has not been pursued.

The cytoplasm of the interstitial cells of the foetal sheep contains no glycogen, glycolipid, or glycoprotein masses, i.e. no PAS-positive granules. This contrasts with the cytoplasm of the interstitial cells of poikilotherms (Cavazos and Melampy, 1954), which contains glycogen, and with that of adult homiotherms, which contains diastase-resistant polysaccharide/protein complexes (Montagna and Hamilton,1952). On the basis of these findings it appears that glycogen is entirely absent from the foetal and adult homiothermal interstitial cell; the prominent red, refractile, diastase-resistant granules to be seen in the interstitial cell of the adult ram (Cavazos and Melampy) must make their appearance some time after birth.

In common with the sparrow, chaffinch, greenfinch, mouse, and Leghorn cockerel (Lofts and Marshall,1956), rat

(Lynch and Scott,1951), deer (Wislocki,1949), and man (Mancini,Nolazeo and Dalze,1952) the interstitial cells of the foetal sheep contain sudanophil material. The material occurs in the form of very fine droplets which are only a fraction of the size of the corresponding droplets in interstitial cells of mice.

No Schiff-positive lipids occur in the interstitial cell of the foetal sheep. Similarly, these lipids are absent from the interstitial cells of neonatal mice, cold-stressed mice, the adult rat (Albert and Leblond, 1946), and the adult deer (Wislocki,1949). It would appear that Schiff-positive lipids do not occur in the foetal testes of species so far investigated; they are present in large amounts in the interstitium of most adolescent animals so far studied; they are usually present in reduced amounts in the corresponding adult testes, although some adult testes contain no demonstrable plasmalogens. In addition to age and species variation, climatic factors also influence the amount of interstitial cell plasmalogen.

While the above histochemical findings suggest that interstitial cell androgens are present in small quantities, if at all, in the foetal sheep, androgen has been demonstrated in the testis of the foetal bull (Moore,1950), and it has recently been shown (Price and

Pannabecker,1956) that the foetal rat testis in vitro produces demonstrable androgens. It is difficult to reconcile these observations with the classical view that the male secondary sexual organs require merely an initial androgenic stimulus for their differentiation, and that, for a time thereafter, their development is independent of the male sex hormone (Moore,1950).

The author is grateful for the research facilities provided in the Department of Anatomy at Glasgow University.

REFERENCES

ALBERT,S., and LEBLOND, C.P., 1946. Endocrinology, 39, 386.

ALLEN, B.M., 1903. Amer. J. Anat., 3, 89.

BASCOM, K.F., 1923. Amer. J. Anat., 31, 233.

CARLETON, H.M., and DRURY, R.A.B., 1957. Histological technique. Oxford (University Press).

CAVAZOS, L.F., and MELAMPY, R.M., 1954. Amer.J. Anat., 95, 467.

DEMPSEY, E.W., 1948. Rec. Prog. Hor. Res., 3, 127.

GILLMAN, J., 1948. Carnegie Contrib. Embryol., 210, 81.

JOST, A., 1956. Ciba Foundation Colloquia on Ageing, 2, 18.

LOFTS, B., and MARSHALL, A.J., 1956. J. Endocrin., 13, 101.

LILLIE, R.D., 1954. Histopathologic technic and practical histochemistry. New York (Country Life Press).

LYNCH, K.M., and SCOTT, W.W., 1951. Endocrinology, 49, 8.

MANCINI, R.E., NOLAZKO, J., and BALZE, F.H., 1952. Anat. Rec., 114, 127.

MONTAGNA, W., and HAMILTON, J.B., 1952. Ibid., 112, 237.

MOORE, C.R., 1950. J. clin. Endocrinol., 10, 942.

PRICE, D., and PANNABECKER, R., 1956. Ciba Foundation Colloquia on Ageing, 2, 3.

ROOSEN-RUNGE, E.C., and ANDERSON, D., 1959. Acta Anat., 37, 125.

VALLANCE-OWEN, J., 1948. J. Path. Bact., 60, 325.

WISLOCKI, G.B., 1949. Endocrinology, 44, 167.

OBSERVATIONS ON THE GROWTH AND HISTOCHEMISTRY OF THE LEYDIG TISSUE IN THE POSTNATAL PREPUBERTAL MOUSE TESTIS.

J. Anat. Lond. (1961), 95, 3, p.p. 357-370.

Two reasonably distinct generations of Leydig cells have been described in most mammalian species so far studied: the first is a foetal generation, while the second is usually described as arising around puberty. Following Gillman (1948) it is now widely accepted that the foetal Leydig cell arises from a mesenchymal cell. During cytomorphosis the oval nucleus of this precursor becomes rounded and vesicular, while the chromatin becomes localized just inside the nuclear membrane. Thereafter the cytoplasm increases in amount, its processes diminish in size and number and the cell body assumes a polygonal epithelioid appearance. Lipid droplets appear at a variable point during this process.

In man and horse (Gillman,1948) foetal Leydig cells are supposed to reach a developmental maximum at or about mid-term; thereafter the Leydig tissue undergoes a regression, the occurrence and timing of which has not been satisfactorily explained. By contrast in the rat (Roosen-Runge & Anderson,1959), rabbit (Allen,1904) and pig (Bascom & Osterud,1927) similar Leydig tissue maxima do not occur until birth. The ensuing regression in these animals has been ascribed to deprivation of maternal oestrogens and gonadotrophins. The Leydig cell of the foetal mouse has not been investigated in this connexion.

The intertubular tissue of the postnatal, prepubertal testis in most mammalian species, for example the bull (Hooker, 1944) is said to show little evidence of differentiation and consists mainly of mesenchymal cells. Many of these cells, however, contain typical Leydig nuclei (Hooker,1948). While it is widely accepted that the second, or postnatal, generation of Leydig cells makes its first appearance at puberty or at least undergoes a tremendous increase in cell number about that time, Hooker has pointed out that this generalization is based on surprisingly few observations.

Close scrutiny of the literature on the life history of the Leydig cell indicates that our knowledge is, at the best, fragmentary, and many aspects remain to be explored. The present investigation was undertaken in an attempt to ascertain whether there are two entirely separate generations of Leydig cells, foetal and pubertal, and if so, whether the pubertal generation in fact arises as the result of massive cell division or cell differentiation. Furthermore , it was hoped to establish whether or not a fully differentiated Leydig cell can undergo mitosis. Finally the relationship of histochemical changes in the Leydig cell to its androgenic function during the prepubertal period requires clarification.

MATERIAL AND METHODS

The work may conveniently be divided into two parts: (1) a description of the histology and histochemistry of the pre-pubertal testes and seminal vesicles; (2) observations of colchicine arrested mitoses in pre-pubertal and adult testes.

The animals used were albino mice aged from 0 days to 8 weeks; thirty-three mice were sacrificed in the preparation of the age series, twenty-two were used in the colchicine study. Puberty, in the mouse, occurs about the fourth week of extra-uterine life but differentiation of the secondary sexual organs is not complete, however, until the eighth week of postnatal life. For this reason cytological and cytochemical studies were extended to include animals up to 8 weeks old notwithstanding the fact that the testis reaches its adult size by the end of the sixth week of extra-uterine life.

In preparation of the age series the mice were killed at 0, 7, 14, 21, 28, 35, 42, 49 and 56 days. Two testes from each age group were subjected to each of the following procedures:

(1) A modified McManus/Hotchkiss periodic acid-Schiff (PAS) reaction (Baillie,1960a) which minimizes testicular shrinkage and distortion of Leydig tissue.

(2) Haematoxylin and eosin staining of sections prepared for the P.A.S. procedure.

(3) Coloration with Sudan black to demonstrate total lipids present after formol calcium fixation.

(4) Haye's modification of Feulgen and Voit's plasmal reaction (Lillie,1954a) to show acetal phosphatides and, possibly, steroids (Dempsey,1948).

(5) Staining with 2:4-dinitrophenyl hydrazine (Albert & Leblond,1946).

(6) Fixation in 70% cold ethanol for demonstration of alkaline phosphatase (Lillie,1954b).

One testis from each of the first six arbitrary age groups was also stained for mitochondria after fixation in Helly's fluid.

Testicular volume was measured on gonads fixed in formol calcium using the formula $V = 4/3\pi b^2 a$, where V = testicular volume, b = half the equatorial diameter of the testis and a = half its polar diameter (Harrison & MacMillan,1954). The measurements were carried out under a microscope using a scale calibrated in $\frac{1}{100}$ mm. In common with the observations of Harrison & MacMillan (1954) it was found that measurements on the unfixed testis were inaccurate since the gonad flattens when placed on a hard surface: agitation during the initial stages of fixation obviates this source of error.

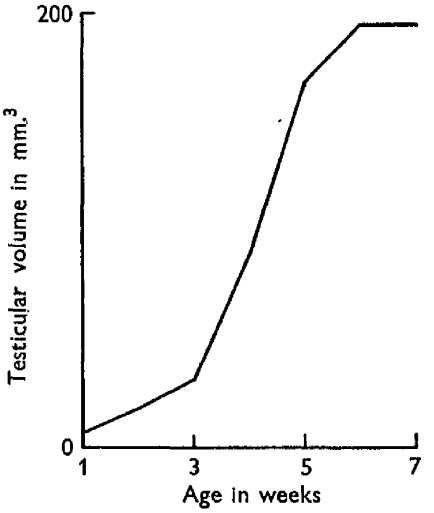
Preliminary work indicated that there was gross Leydig tissue shrinkage and distortion after alcoholic fixation (cf.Pl.1,fig.2 and Pl.3,fig.9) and, moreover, relatively large testes (e.g.those of the adult hedgehog and rat) were frequently irregularly fixed in their central portions. Formalin fixed mouse testes were largely free of these artefacts.

At the end of the fourth week of extra-uterine life, when the seminal vesicles became large enough for weighing, they were dissected out, their outlets clamped and the entire preparation fixed in formol corrosive solution for 6 hr. and then weighed. Since fluctuations in androgen release are thought to be reflected by altered secretory activity in the seminal vesicles (Baker, Schairer,Ingle & Li 1950), it was considered advisable to preserve all of the secretion for weighing. On this basis it was felt that attempts to weigh the fresh vesicle were unsatisfactory due to partial loss of secretion owing to agonal contraction, and that the weight of the fixed vesicles provided a truer figure for comparison. Vesicles were routinely stained for alkaline phosphatase and with haematoxylin and eosin.

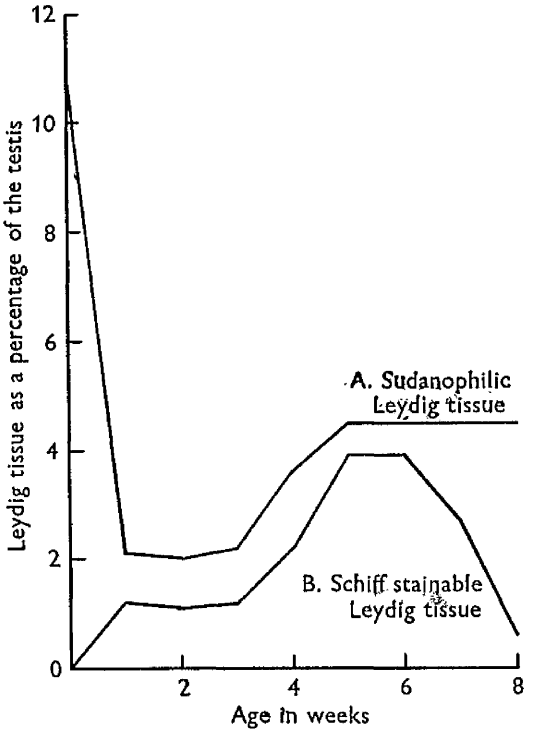
All mature Leydig cells were found to have sudophilic cytoplasm. Numerous random sections of testes from each age group coloured with Sudan black were projected by

microscope on to paper, when the areas of black tissue (i.e. Leydig cells) were marked out by pen. The relative volume of Leydig tissue was derived as a percentage by weighing the total paper field and, later, the cut-out areas representing Leydig tissue. The total volume of Leydig tissue was calculated from the percentage and the testicular volume. In a similar fashion the volume of Leydig tissue containing plasmalogens (Schiff stainable lipids; acetal phosphatides and possibly steroids) was also calculated. The relative and total volumes of intertubular tissue were estimated on P.A.S. stained material.

Twenty-two animals were used in the colchicine study, being sacrificed in pairs between birth and the end of the eighth week of postnatal life: in addition, four adult animals were surveyed. Each animal received an intra-peritoneal injection of colchicine (0.1 mg. in water/100 g. body weight) at 10.00 a.m. on the appropriate day. They were killed 5 hr. later and their testes excised for examination using the PAS technique. The period was identical with that used by Ebling (1954) and Bullough (1950) who selected it to vitiate the effects of the diurnal mitotic cycle. The mitotic rate was calculated by counting the total number of typical Leydig cells in a given field and also the number of Leydig cells in the same



Text-fig. 1. The testicular growth curve is sigmoid in form and growth is complete by the end of the sixth week of postnatal life.



Text-fig. 2. The sudanophilic Leydig tissue, when portrayed as a percentage of the testis, is apparently reduced in volume during the first week of extra-uterine life and remains at a low level for 3 weeks before undergoing an apparent increase to reach the adult level. Neonatal Leydig tissue has no Schiff stainable lipids. Thereafter the relative volume of Schiff positive Leydig tissue behaves in the same fashion as that of the sudanophilic Leydig tissue until the seventh week when Leydig cell plasmalogens are strikingly reduced.

field whose nuclei were in arrested mitosis: from these figures the mitotic rate is derived as a percentage per 24 hr.

RESULTS

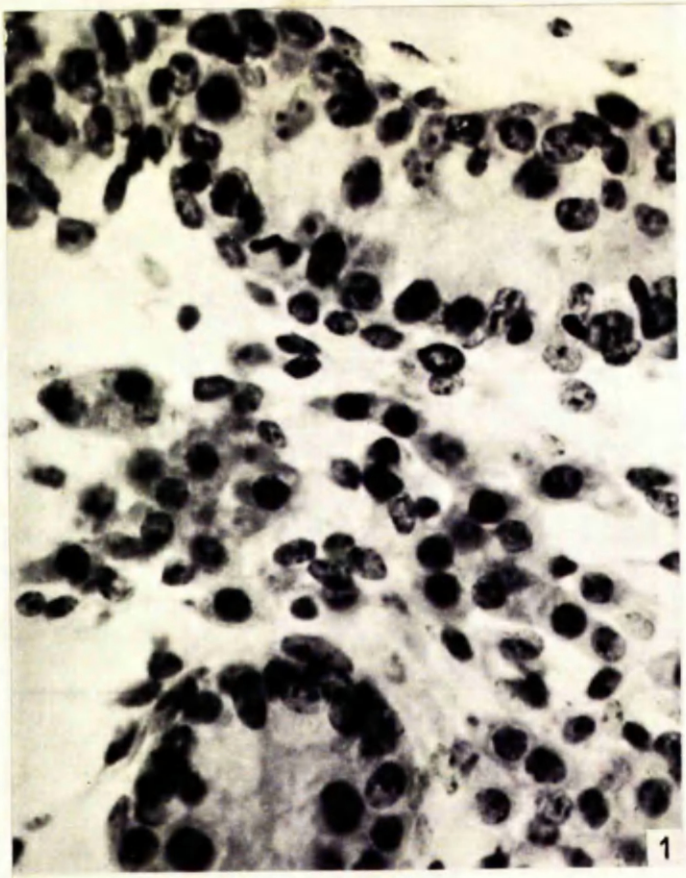
(1) Testicular volume

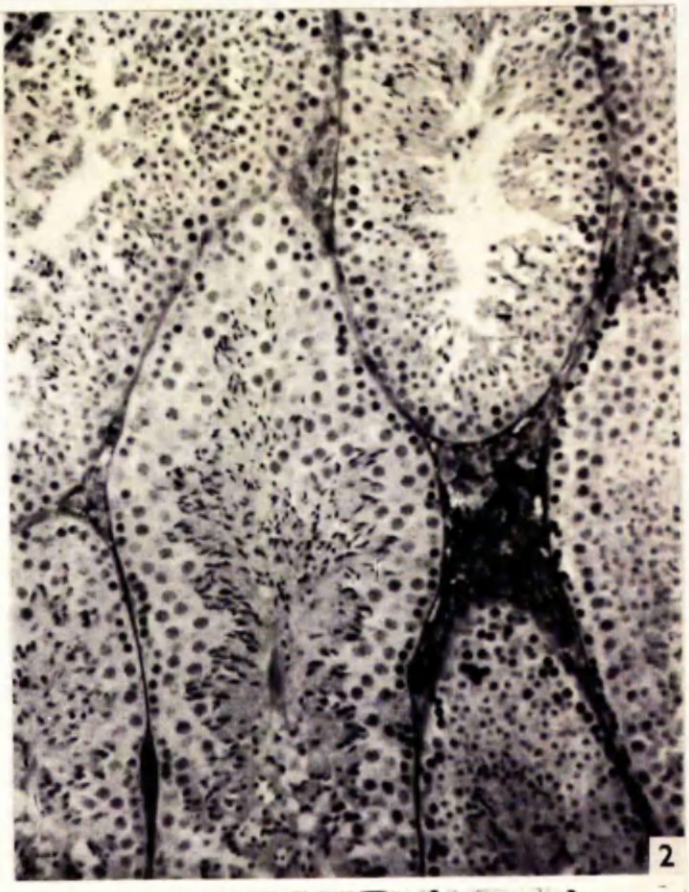
As will be seen from Text-fig. 1, the testicular growth curve is sigmoid in form. Growth is completed by the time the animal is 6 weeks old.

(2) Haematoxylin and eosin observations

At birth the intertubular tissues account for over 70% of the testis. Typical, stellate or spindle-shaped mesenchymal cells, exhibiting occasional mitotic figures, are linked by transitional forms with peritubular fibroblasts on the one hand and Leydig cells on the other. The Leydig cells usually occur in groups or clumps. Their cytoplasm is abundant, rounded or hexagonal in shape, strongly eosinophilic (Pl.1, fig.1), and is not vacuolated. The cell membrane is clearly defined. The eccentric nucleus is round to ovoid and its membrane is lined with chromatin which also forms four or five heavily staining granules in the interior.

At the end of the first week, the intertubular tissue is seen to be apparently much reduced, the Leydig cells are less readily visible, and their cytoplasm has become vacuolated. This apparent atrophy is still evident at the





EXPLANATION OF PLATES

Plate 1

Fig. 1. Neonatal testis, H. & E. Note the abundant eosinophilic Leydig cytoplasm. × 350.

Fig. 2. Testis at 7 weeks, PAS. Epithelioid Leydig cells stand out prominently in the intertubular spaces: the extracellular spaces contain PAS positive ground substance. × 150.

Fig. 3. Neonatal testis, Sudan black. Note relative prominence of Leydig tissue. × 90.

Fig. 4. Testis at 7 days, Sudan black. The intensity of Leydig sudanophilia has increased, but the amount is relatively reduced. × 90.

end of the second week, but during the third, fourth and fifth weeks the Leydig cells became much more prominent. Their vacuolated foamy cytoplasm contains small numbers of minute yellow refractile granules which appear to increase in number as the animal approaches maturity. There is no significant change during the sixth, seventh and eighth weeks.

(3) Periodic acid-Schiff reaction

The neonatal Leydig cell cytoplasm does not stain with the PAS reaction. A few cells have PAS positive material either lining part of the cell membrane or forming four or five juxta nuclear granules. The staining propensity of this material is unaffected by treatment with diastase or with the lipid solvents. A few peculiar cells with pycnotic nuclei and scanty cytoplasm, which is strongly PAS positive, are to be seen. There is no demonstrable PAS positive, intercellular ground substance.

In testes taken from older animals the individual Leydig cells show a constant picture: their foamy cytoplasm is very faintly PAS positive in sharp contrast with the strongly PAS positive gound substance. Quite a few cells contain numerous bright red, diastase-resistant, refractile granules. The shrunken cells with pycnotic nuclei and PAS positive cytoplasm are fairly common in

the 7-day-old testis, but they are rarely visible in older gonads. As the mice mature the connective tissue ground substance becomes clearly defined by the PAS procedure and contains PAS positive material which resists digestion with amylase and extraction with chloroform (Pl.1, fig.2). The individual Leydig cells are separated by ground substance which in places contains large oval or round spaces.

(4) Sudan black observations

At birth (Pl.1,fig.3) the mouse testis interstitium contains abundant quantities of sudanophilic material, which on closer inspection, is seen to consist of numerous minute lipid droplets dispersed through the Leydig cytoplasm. The cell nucleus is rarely obscured by the masses of lipid droplets and the cell boundaries are prominently defined. Undifferentiated mesenchymal cells have no visible lipids: transitional forms are common. The testes of 7-, 14- and 21-day-old mice present a uniform picture (Pl.1,fig.4). The amount of interstitial sudanophilic material has undergone a marked apparent reduction (c.f.Text-fig.2), and the individual cells are rounded or polygonal. Transitional forms, are as before, still visible.

The interstitium of later testes (from mice aged 4, 5,6,7 and 8 weeks) is similar (Pl.2,fig.5): the lipid

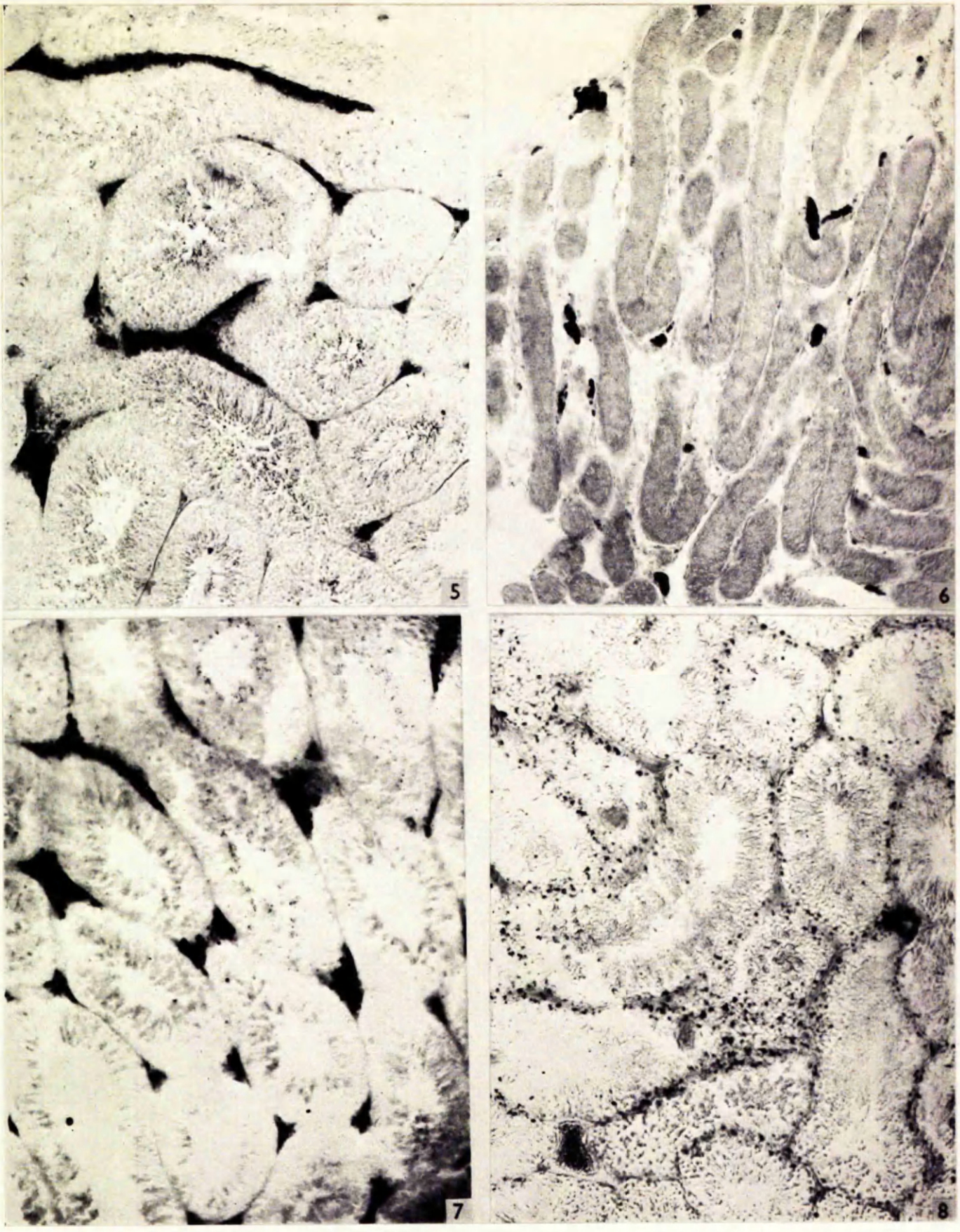


PLATE 2

Fig. 5. Testis at 6 weeks, Sudan black. There is a relative increase in sudanophilic Leydig tissue. ×90.

Fig. 6. Testis at 7 days, the plasmal reaction. Interstitial plasmalogens have just appeared. ×90.

Fig. 7. Testis at 6 weeks, the plasmal reaction. Note the prominence of Leydig cell plasmalogens.

Fig. 8. Adult testis, the plasmal reaction. Leydig plasmalogens are greatly reduced; Sertoli plasmalogens have now appeared. ×90.

laden Leydig cells have undergone a marked relative increase and are strikingly prominent.

(5) The plasmal and phenyl hydrazine reactions

The neonatal Leydig cells contain no Schiff stainable lipids in contrast to all subsequent age groups. Sections of 7-, 14- and 21-day old gonads are alike (Pl.2,fig.6) when exposed to Schiff's reagent. Many groups of Leydig cells stain an intense purple colour and their cytoplasm contains swarms of minute purple-coloured droplets which tend to obscure details of the cell nucleus and boundaries. During the fourth and fifth weeks of postnatal life the number of cells in the interstitium with Schiff stainable lipids undergoes a remarkable increase which is maximal at the end of the sixth week (Text-fig. 2; Pl.2,fig.7); Leydig cell plasmalogens are then remarkably reduced in the ensuing 2 weeks, reaching the characteristic low adult level (Pl.2,fig.8) by the end of the eighth week. While this striking reduction in Leydig cell plasmalogen is occurring, abundant Schiff stainable lipids make their appearance in the Sertoli cells of the seminiferous tubules. This lipid (and lipid it must be since it dissolves in the fat solvents) does not colour with Sudan black. 2:4-Dinitrophenyl hydrazine gives the same results as Schiff's reagent in the testis.

Table 1. *Mensural data of testes and seminal vesicles together with an analysis of intertubular and Leydig tissues*

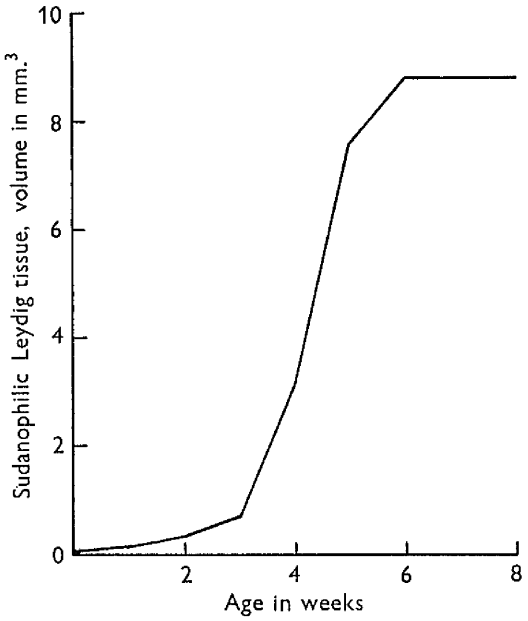
Age in days	0	7	14	21	28	35	42	49	56
Testicular volume (average) in mm.^{3}	0·8	6·1	18·3	31·7	88·9	169·2	195·4	196·1	196·1
Testicular volume (range) in mm.^{3}	0·6–1·0	5·7–6·4	17·6–19·0	31·0–32·4	88·1–90·0	168·1–170·0	194 4–196·0	195 5–197·0	195·5–196·8
Polar diameter (average) in mm.	1·68	3·02	4·4	5·25	7·68	9·9	9·9	9·9	9·9
Equatorial diameter (average) in mm.	0·95	1·97	2·82	3·4	4·7	5·86	6·14	6·15	6·15
Seminal vesicle weight (average) in mg.	—	—	—	—	12	25	112	218	230
Seminal vesicle weight (range) in mg.	—	—	—	—	10–13	21–28	107–116	204–230	220–245
Logarithm of seminal vesicle weight	—	—	—	—	1·0792	1·3979	2·0492	2·3385	2·3617
Intertubular tissue, %	71·3	17·0	6·1	4·1	5·3	5·5	5·3	5·4	5·4
Sudanophilic Leydig tissue, %	10·6	2·1	2·0	2·2	3·6	4·5	4·5	4·5	4·5
Sudanophilic Leydig tissue, volume in mm.^{3}	0·08	0·13	0·37	0·7	3·2	7·6	8·8	8·8	8·8
Logarithm of sud. Leydig tissue volume	$\bar{2}$·9031	$\bar{1}$·1139	$\bar{1}$·5682	$\bar{1}$·8451	0·5051	0·8808	0·9445	0·9445	0·9445
Schiff stainable Leydig tissue, %	0	1·2	1·1	1·2	2·2	3·9	3·9	2·7	0·6
Schiff stainable Leydig tissue, volume in mm.^{3}	0	0·07	0·20	0·38	1·96	6·59	7·6	5·28	1·18
Leydig mitotic rate/24 hr., %	6·7	6·2	5·3	6·7	6·7	6·2	1·1	0·1	0·0

(6) Quantitative histological observations

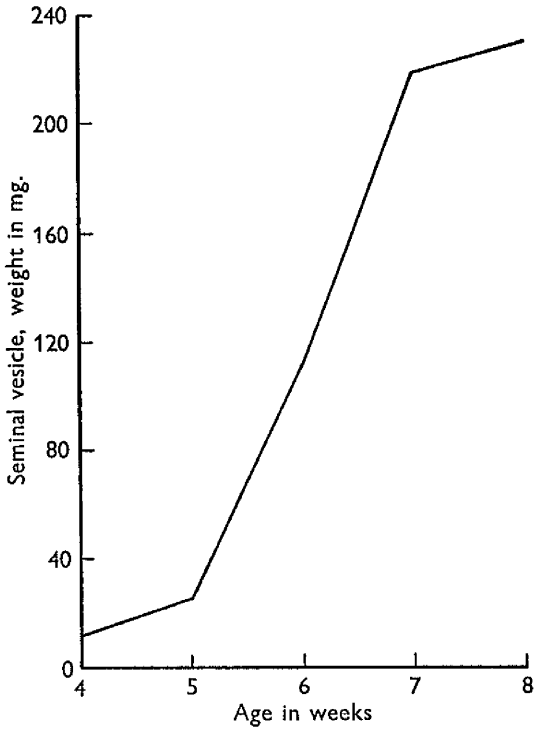
The quantitative data are summarized in Table 1. The fluctuations in the relative volumes of sudanophilic Leydig tissue and of Schiff positive Leydig tissue are shown in Text.fig.2. From the testicular volume at the appropriate age (Table 1) the relative volumes of sudanophilic and of Schiff positive Leydig tissue can be translated into absolute volumes (Text-fig.3 and Table 1).

From Text-fig.2 we see that the sudanophilic Leydig tissue undergoes a marked apparent reduction in volume during the first week of extra-uterine life. It remains at this low level for 3 weeks before undergoing an apparent increase to reach the adult level. No Leydig tissue contains Schiff stainable lipids in the neonatal testis. Thereafter the relative volume of Schiff positive Leydig tissue behaves in much the same fashion as that of the sudanophilic Leydig tissue, although at any given time the volume of Schiff positive Leydig tissue is appreciably less than that of sudanophilic Leydig tissue. During the seventh and eighth weeks of postnatal life Leydig cell plasmalogens are strikingly reduced.

Reference to Text-fig.3 indicates that while fluctuations take place in the relative Leydig tissue volume, the absolute volume shows a continuous regular increase. The growth curve is sigmoid in form.



Text-fig. 3. In contrast to the relative volume (Text-fig. 2), the absolute volume of sudanophilic Leydig tissue undergoes a continuous regular increase between birth and adult life. The growth curve is sigmoid in form.



Text-fig. 4. The seminal vesicle weight increases rapidly between the fourth and seventh weeks of postnatal life and the growth curve is sigmoid in form.

(7) Alkaline phosphatase observations

The neonatal testis contains no demonstrable alkaline phosphatase; this enzyme makes its appearance in the fibroblast sheath which surrounds the basement membrane of the seminiferous tubules at the end of the third week. It is thereafter a prominent characteristic of the basement membranes of the blood vessels and of the seminiferous tubules. The Leydig cells never contain alkaline phosphatase (Pl.3,fig.9).

(8) Mitochondria

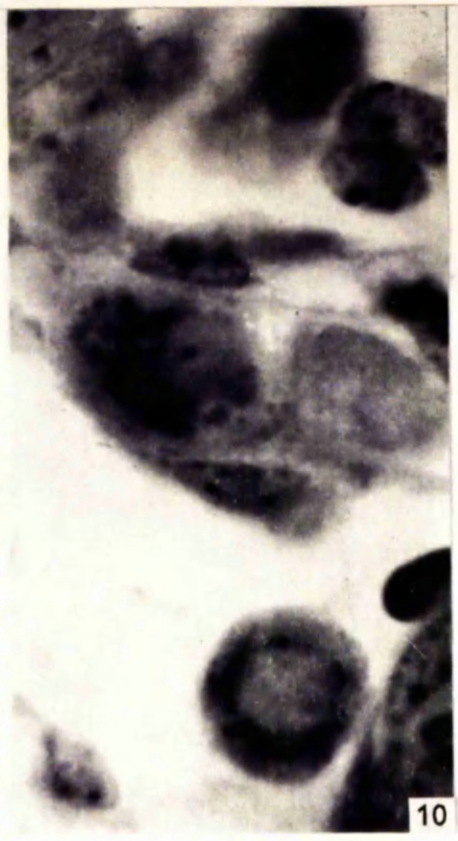
Throughout the age series mitochondria are visible as minute round refractile granules which fill variable areas of cytoplasm: the perinuclear area is devoid of mitochondria. Many cells are vacuolated,mitochondria being absent from the vacuolated region.

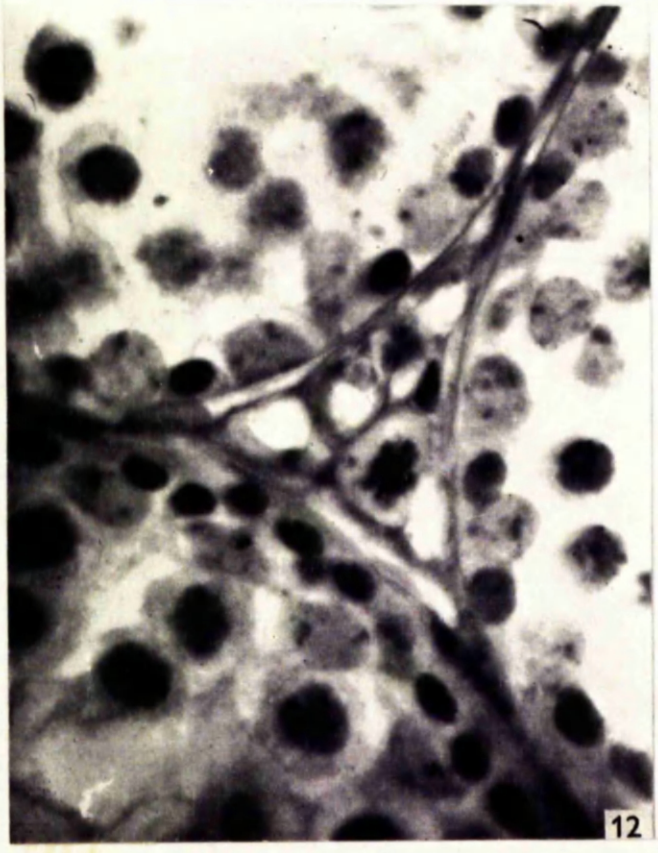
(9) Seminal vesicle weight

The salient features are summarized in Table 1 and in the sigmoid growth curve portrayed in Text-fig.4. The vesicles are not large enough for weighing until the animal is 4 weeks old; growth thereafter is largely completed by the end of the seventh week of extra-uterine life.

(10) Seminal vesicle cytology

During the initial period of growth, between the fourth and sixth weeks of post-natal life, the principal





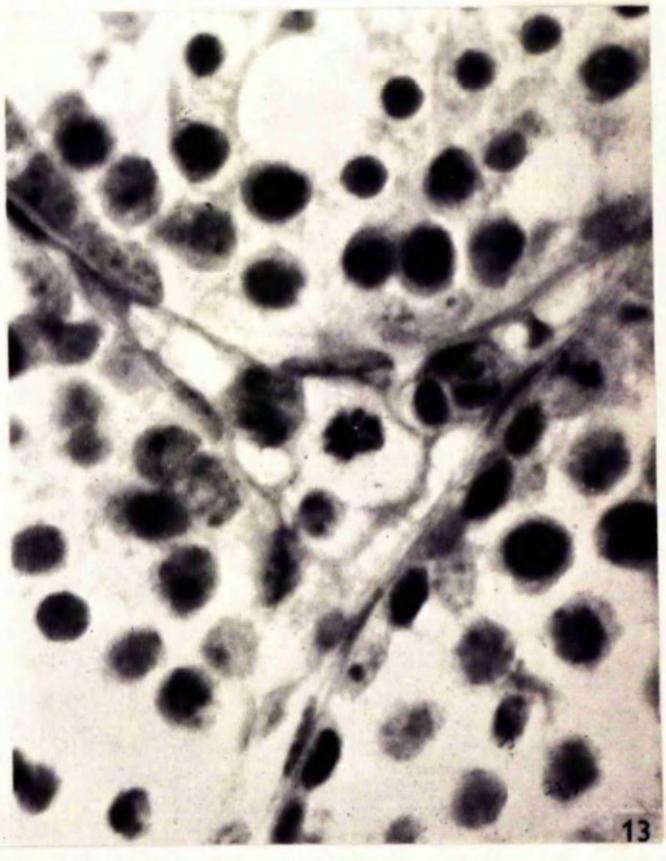


PLATE 3

Fig. 9. Testis at 6 weeks, alkaline phosphatase. The basement membranes of the seminiferous tubules are prominently stained. Note the gross interstitial shrinkage produced by alcoholic fixation. Leydig tissue contains no alkaline phosphatase. × 150.

Fig. 10. Neonatal testis, acid fuchsin and toluidine blue. Leydig cells contain numerous granular mitochondria. × 950.

Fig. 11. Seminal vesicle at 4 weeks, alkaline phosphatase. The stroma contains a large amount of the enzyme. × 150.

Figs. 12 and 13. Testis at 3 weeks, PAS. Colchicine arrested Leydig mitoses are readily visible. × 950.

changes which take place are proliferation of the connective tissue stroma, an increase in the height of the columnar epithelium lining the organ, and the appearance of alkaline phosphatase in the stroma and in the basement membrane of the epithelium (Pl.3,fig.11). It seems likely that the later increases in seminal vesicle weight, between the sixth and eighth weeks, are mainly due to the storage of an eosinophilic secretion in the lumen and medial ramifications, or diverticula, of the vesicle. Failure to fix the gland before weighing results in the vesicle presenting a collapsed shrunken appearance suggestive of little secretory activity on account of the extrusion of secretion.

(11) Results of colchicine administration

It was found that mice seven days old tolerated the stated dose poorly; in all some five animals of this age were sacrificed before two were found which survived a five hour period. All other age groups seemed unperturbed by the administration of colchicine. Mitotic figures are readily seen in typical Leydig cells following colchicine administration. To facilitate comparison with the daily increment in Leydig tissue volume, the calculated Leydig mitotic rate is expressed as a percentage (Table 1).

The daily mitotic average during the first 5 weeks of postnatal life works out at 6.3%. Thereafter it falls

progressively: no mitotic figures were seen in adult testes. An example of Leydig mitotic activity is to be seen in Pl.3,figs.12 and 13.

DISCUSSION

The mouse Leydig cell is morphologically typical of the mature mammalian Leydig cell having abundant eosinophilic cytoplasm and a rounded vesicular nucleus. The minute granular mitochondria correspond in shape and size with those described by Rasmussen (1932) in the Leydig cell of the cat, mouse and guinea-pig. They are not, however, restricted to the peripheral cytoplasm as suggested by Fawcett & Burgos (1956). The shape, size and distribution of mitochondria is unaffected by age or alterations in lipid distribution. The cytomorphosis of Leydig cells from mesenchymal cells involves the progressive acquisition of mitochondria.

When studied using routine H. & E. staining the Leydig cells are prominent at birth, difficult to discern during the ensuing 2 weeks and become prominent again at the onset of sexual maturity thereby supporting Hooker's (1948) statement that: 'In most species the intertubular tissue of the postnatal, prepubertal testis, shows little differentiation.' The use of the PAS technique however, renders Leydig cells not only visible, but prominent during the entire postnatal prepubertal period,

indicating continuity of the supposed foetal and pubertal generations. Moreover, reference to Text-figs.2 and 3 indicates that, while relative fluctuations in the Leydig tissue volume take place, throughout the postnatal prepubertal period, the absolute volume shows a continuous regular increase. From these facts one must conclude that there is only one generation of Leydig cells in the postnatal mouse and that the concept of separate foetal and pubertal generations of Leydig cells is based on inadequate histological methods for demonstrating the relatively slowly growing Leydig tissue in the rapidly expanding prepubertal testis.

When stained with the PAS procedure, mouse Leydig cytoplasm stains a pale pink colour and may contain a number of minute red refractile glycoprotein granules. This corresponds with the view (Baillie, 1960b) that homoiothermal vertebrate Leydig cells contain carbohydrate-protein complexes acquired after birth, but no glycogen. The Leydig cells of poikilotherms, on the other hand, contain visible glycogen granules. Peculiar shrunken crenated cells with pyonotic nuclei and intensely PAS positive cytoplasm are to be seen in the intertubular spaces of the mouse testis. They are similar to the atrophic cells described in the inter-tubular tissue of the foetal sheep testis (Baillie,1960b).

In common with the Leydig cells of the sparrow, chaffinch, greenfinch and Leghorn cockerel (Lofts & Marshall, 1956), deer (Wislocki,1949) and man (Montagna & Hamilton,1952; Mancini,Nolazco & Balze,1952), the mouse Leydig cells contain abundant droplets of sudanophilic lipid. This contrasts with some species of rat (Lofts & Marshall,1956; Lynch & Scott,1951) which possess no sudanophilic material in their intertubular tissue. During differentiation from its mesenchymal precursor the Leydig cell acquires minute droplets of lipid which coalesce progressively giving rise to the large granular masses characteristic of mature Leydig cells. At all times in the prepubertal testes fully differentiated Leydig cells with sudanophilic cytoplasmic inclusions exist, further evidence against the widely held concept of separate foetal and pubertal Leydig cell generations discussed above. There is no qualitative change in the sudanophilic interstitial elements which can be correlated with the onset of sexual maturity.

The observations of Albert & Leblond (1946) on the reactivity of the mouse Leydig tissue to Schiff's reagent and 2:4-dinitrophenyl hydrazine have been confirmed with two reservations; first, the neonatal testis contains no complex lipids stainable with these reagents and secondly the mature animal's Leydig cells contain much less of

these materials than do the immature animal's cells (Pl.2,figs.6-8). The distribution of plasmalogens appears to depend on age and species factors and is also influenced by environmental conditions (Baillie,1961).

In contrast to human Leydig cells (Mancini et al. 1952) and to the Leydig cell of rutting deer (Wislocki, 1949) the mouse Leydig cell is devoid of alkaline phosphatase at all stages of its development.

It is generally accepted (Burrows,1949) that seminal vesicle weight alterations reflect accurately fluctuations in androgen release by the testis, despite Hooker's (1942) suggestion that tissue sensitivity to circulating androgen is raised at puberty. Seminal vesicle growth appears to be unrelated to any morphological or histochemical events occurring in the seminiferous tubules; the appearance of Sertoli cell plasmalogens coincides approximately with the end of seminal vesicle growth, and is thus unlikely to bear any relation to androgen synthesis.

On the other hand, from Text-fig.3 it will be seen that the total volume of Leydig tissue undergoes a regular increase during extra-uterine life. This increase is completed by the end of the sixth week and is seen to antedate, if compared with Text-fig.4, a comparable seminal vesicle growth pattern. From the similarity of these two graphs, together with their positions relative to the time

axis, it would seem that the growth of the androgen dependent tissue might be related to the growth in volume of Leydig tissue.

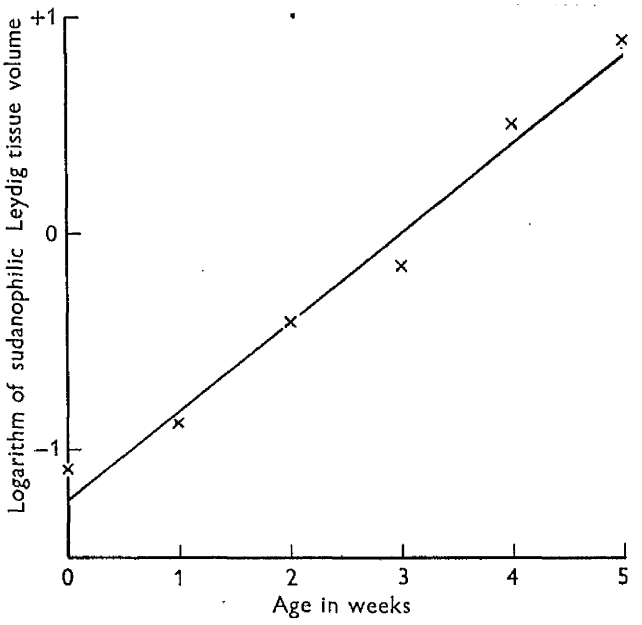
This impression is strengthened when one compares the corresponding log graphs (Text-figs.5 and 6), which are corrected for standard error, and finds that the gradients, which are indices for the growth rates of the two tissues, are similar.

It is notable that the striking reduction in Schiff stainable lipids during the seventh and eighth weeks of postnatal life has no effect on the regularity of seminal vesicle growth. Since Schiff's reagent stains phosphatides and, theoretically at least, steroids but not neutral fats, it is probable that Leydig's plasmalogens are in some way related to androgen metabolism. Androgen production includes synthesis from cholesterol or acetate (Heard et al. 1956) storage, and release into the blood stream. The varying levels of Schiff stainable lipids in the Leydig tissue may reflect variations in the amount of hormone or its precursors being stored at different periods. Similar phenomena have been described in the adrenal of the hibernating ground squirrel (Zimmy,1959) and also in the testes of cold stressed adult mice (Baillie,1961).

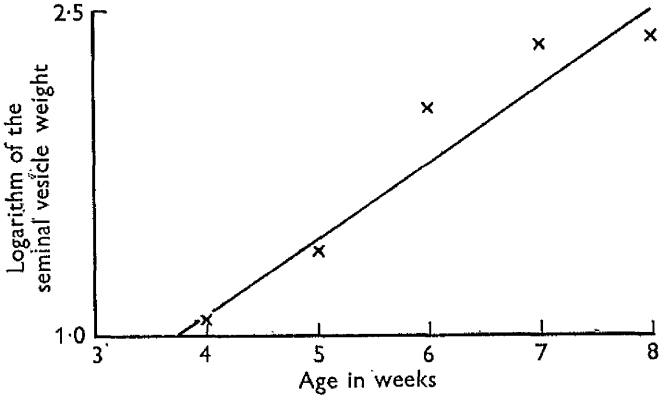
Mitotic figures in the postnatal Leydig cell have only been described in inflammatory reactions and in tissue

culture experiments (Rasmussen,1932) though they are numerous in foetal material (Roosen-Runge & Anderson, 1956). In the immature mouse the use of colchicine indicates that fully differentiated typical Leydig cells can and do divide under physiological conditions: This does not appear to take place to any observable extent in the normal adult mouse Leydig cell. Studies on senescent mice have yet to be made.

From Text-fig.5 it will be seen that the logarithm of the Leydig tissue volume (as calculated from Sudan black sections) undergoes a uniform regular increase of 14.5% ± 1% per day between birth and the end of the fifth week. In other words during the prepubertal period the absolute volume of Leydig tissue increases at a compound rate of roughly 15% per day. If this daily increment is due to formation of new Leydig cells, then one might expect daily, for every 100 existing cells either fifteen transitions from mesenchyme or fifteen mitotic figures. Since the average daily mitotic rate during the first 5 weeks of postnatal life is 6.3%, the increase in the Leydig tissue volume is due both to Leydig cell division and to recruitment from mesenchyme in approximately equal proportions, provided that existing morphologically mature Leydig cells do not materially increase in size: if cell size does increase then the mesenchymal contribution must



Text-fig. 5. The sudanophilic Leydig tissue volume grows at a rate of 0·0587 log units per day (14·5 %) between birth and the end of the fifth week: the standard error of the straight line is ±0·004 log units per day (±1 %).



Text-fig. 6. The seminal vesicle weight increases at a rate of 0·05 log units per day (12·2 %) between the fourth and eighth weeks of postnatal life: the straight line carries a standard error of ±0·087 log units (±2 %) per day.

be proportionately reduced.

SUMMARY

1. The testes of thirty-three albino mice, aged from 0 days to 8 weeks, have been examined at 7-day intervals: stains used include H. & E., the PAS reaction, Sudan black, 2:4-dinitrophenyl hydrazine, the plasmal reaction, a mitochondrial stain and an alkaline phosphatase technique. Seminal vesicles were also examined using H. & E. and the alkaline phosphatase technique: seminal vesicle weight has been taken as an indicator of androgen production.

2. The concept of separate foetal and pubertal generations of Leydig cells is thought to be based on inadequate histological methods for demonstrating the relatively slowly growing Leydig tissue in a rapidly expanding prepubertal testis and is erroneous in the mouse at least.

3. The mouse Leydig cell, in common with those of other homoiothermal vertebrates, contains glycoprotein; in contrast to the Leydig cell of poikilotherms it has no glycogen.

4. The mouse Leydig cell has sudanophilic lipids; lipids stainable with 2:4-dinitrophenyl hydrazine and Schiff's reagent are absent from the neonatal Leydig cell, present in large quantities in the prepubertal Leydig

cell and present in reduced amounts in the adult cell.

5. Cytomorphosis of the Leydig cell from its mesenchymal precursor includes acquisition of sudanophilic lipids, Schiff stainable lipids and mitochondria.

6. Graphic representation of the growth rates of the Leydig tissue and the seminal vesicle shows that both tissues grow at a similar rate and that the growth of Leydig tissue antedates the growth of the seminal vesicles.

7. The distribution of plasmalogens has been described and is thought possibly to be related to the varying balance between the production of androgen from cholesterol and acetate and its release into the blood stream.

8. Mitotic figures have been demonstrated in typical Leydig cells. The absolute volume of Leydig tissue during the prepubertal phase increases at a compound rate of about 15% per day: the Leydig mitotic rate is 6.3% per day. The increment in Leydig tissue volume is thus due to cell division plus recruitment from mesenchyme. Leydig cells in the adult testis do not appear to undergo mitosis in physiological circumstances.

REFERENCES

ALBERT, S. & LEBLOND, C.P. (1946). Distribution of reactions in normal, castrated, adrenalectomised and hormonally treated rate. Endocrinology, 39, 386-400.

ALLEN, B.M. (1904). The embryonic development of the ovary and testis of the mammals. Amer. J. Anat. 3, 89-153.

BAILLIE, A.H. (1960a). The biology of the Leydig cell; histochemical and histological changes following high epididymal obstruction. J. Endocrin. 20, 339-344.

BAILLIE, A.H. (1960b). The interstitial cell in the testis of the foetal sheep. Quart.J. Micr. Sci. 101, 475-480.

BAILLIE, A.H. (1961). Histochemical studies of the mouse testis following cold exposure. Scot. med. J. 6, 6-11.

BAKER, B.L., SCHAIRER, M.A., INGLE, D.J. & LI, C.H. (1950). The induction of involution in the male reproductive tract by treatment with andreno-corticotrophin. Anat. Rec. 106, 345-360.

BASCOM, K.F. & OSTERUD, H.L. (1927). Quantitative studies of the testis. III. A numerical treatment of the development of the pig testis. Anat. Rec. 37,63-82.

BULLOUGH, W.S. (1950). Epidermal mitotic activity in the adult female mouse. J. Endocrin. 6, 340-349.

BURROWS, H. (1949). Biological Actions of the Sex Hormones, p.256. Cambridge University Press.

DEMPSEY, E.W. (1948). The chemical cytology of endocrine glands. Recent Progr. Hormone Res. 3, 127-158.

EBLING, F.J. (1954). Changes in the sebaceous glands and epidermis during the oestrous cycle of the albino rat. J. Endocrin. 10, 147-154.

FAWCETT, D.W. & BURGOS, M.H. (1956). Observations on the cytomorphosis of the germinal and interstitial cells of the human testis. Ciba Foundation Colloquia on Ageing, 2, 86-99.

GILLMAN, J. (1948). The development of the gonads in man, with a consideration of the role of foetal endocrines and the histogenesis of ovarian tumours. Contr. Embryol. Carneg. Instn, 210, 81-131.

HARRISON, R.G. & MACMILLAN, E.W. (1954). The effects of high epididymal obstruction upon the Leydig cell volume of the rat testis. J. Endocrin. 11, 89-97.

HEARD, R.D.H., BLIGH, E.G., CANN, N.C., JELLINCK, P.N., O'CONNELL, V.J., RAO, B.G. & WEBB, J.L. (1956). Biogenesis of the sterols and steroid hormones. Recent Progr. Hormone Res. 12, 45-70.

HOOKER, C.W. (1942). Pubertal increase in responsiveness to androgen in male rat. Endocrinology, 30, 77-84.

HOOKER,C.W. (1944). The postnatal history and function of the interstitial cells of the testis of the bull. Amer. J. Anat. 74, 1-37.

HOOKER, C.W. (1948). The biology of the interstitial cells of the testis. Recent Progr. Hormone Res. 3, 173-196.

LILLIE, R.D. (1954a). Histopathologic Technic and Practical Histochemistry, p.316. New York: Garden City, Country Life Press Corp.

LILLIE, R.D. (1954b). Histopathologic Technic and Practical Histochemistry, p.202-203. New York: Garden City, Country Life Press Corp.

LOFTS, B. & MARSHALL, A.J. (1956). The effects of prolactin administration on the internal rhythm of reproduction in male birds. J. Endocrin. 13, 101-106.

LYNCH, K.M. & SCOTT, W.W. (1951). Lipid distribution in Sertoli cell and Leydig cell of rat testis as related to experimental alterations of pituitary - gonald system. Endocrinology, 49, 8-14.

MANCINI, R.E., NOLAZCO, J. & BALZE, F.A. (1952). Histochemical study of normal adult human testes. Anat. Rec. 114, 127-148.

MONTAGNA, W. & HAMILTON, J.B. (1952). Histological studies of human testes. II. The distribution of glycogen and other HIO_4-Schiff reactive substance. Anat. Rec. 112, 237-249.

RASMUSSEN, A.T. (1932). Interstitial cells of the testis. Cowdry's Special Cytology, 3, 1674-1725.

ROOSEN-RUNGE, E.C. & ANDERSON, D. (1959). The development of the interstitial cells in the testis of the albino rat. Acta. Anat. 37, 125-137.

WISLOCKI, G.B. (1949). Seasonal changes in testes, epididymides and seminal vesicles of deer investigated by histochemical methods. Endocrinology, 44, 167-189.

ZIMNY, M.L.(1959). Histological studies of the ground squirrel adrenal following hibernation and cold exposure. Anat. Rec. 135, 279-284.

OBSERVATIONS ON THE GROWTH AND HISTOCHEMISTRY OF THE LEYDIG TISSUE IN THE POSTNATAL PREPUBERTAL MOUSE TESTIS.

II.

J. Anat. Lond. In Press.

The interstitial cells of the testis were first described in 1850 by Leydig who noted masses of epithelioid hexagonal fat laden cells in the intertubular spaces. A trophic effect on the germinal epithelium of the seminiferous tubules was initially postulated for these cells until, in 1903, Bouin and Ancel described them as the "interstitial gland" of the testis, and ascribed an endocrine function to this "gland". They suggested, from experimental evidence, that these cells produced and released hormones controlling the secondary sex characteristics.

In a previous communication (Baillie, 1961) the differentiation and mitotic capabilities of the Leydig cell in the immature animal were studied, and an attempt was made to relate the lipid histochemical changes in the Leydig cell to its androgenic function using the techniques available at that time. Since then more accurate methods of assessing androgen production have been evolved (Lindner & Mann, 1960) and great improvements made in the techniques of cellular localisation of some of the enzymes involved in steroidogenesis (Niemi & Ikonen, 1961). In the light of these advances it was felt that a fresh attempt to relate Leydig cytochemistry to androgen production might be more fruitful.

MATERIAL AND METHODS

The investigation may readily be split into three sections: (1) observations on the changes with age demonstrable in the prepubertal testicular interstitial cell using some of the newer histochemical techniques; (2) an account of concomitant changes in the fine structure of the Leydig cell; (3) a morphological and biochemical assessment of the endocrine activity of the growing testis.

The animals used in the project were male Swiss White mice aged from birth to ten weeks. 572 mice were used in preparation of the histochemistry age series, 8 in the ultrastructure age series, and 80 in compiling the biochemical and morphological data relating to the endocrine function of the testis.

(1) Histochemistry. Ten different techniques were employed and 44 animals were sacrificed in the preparation of each histochemical series, being killed in groups of four at birth and at weekly intervals thereafter up to and including the end of the tenth week of postnatal life. The last three enzyme methods entailed the use of more than one substrate, and each substrate used was treated as a series in its own right (entailing the use of 44 animals) for reasons which will become apparent in the discussion. With the exception of the first two techniques,

all testes used were quenched in solid carbon dioxide and sectioned on a cryostat at 20μ. The following histochemical components were studied:-

(A) Ascorbic acid, using the technique of Bacchus (1950), with the exception that, after silver impregnation, the tissues were dehydrated in cellosolve and impregnated in ester wax (Watford Chemical Co.).

(B) Glucose, with Muller's (1955) method.

(C) Cholesterol, with the reaction described by Okamoto, Shimamoto & Sonoda (1944).

(D) Lipofuscin, using the Schmorl (Pearse, 1960) reaction.

(E)α ketols, with the method of Pearse (1960).

(F) Cytochrome oxidase activity, using the G-Nadi reaction of Moog (1943).

(G) Succinate dehydrogenase activity, by the method of Nachlas et al., (1957), but using Nitro-B T as the coupling agent.

(H) Aryl sulphatase activity, using the technique first described by Rutenburg, Cohen & Seligman (1952). Two substrates were employed, namely, potassium-6-benzoyl-2-naphthyl sulphate, and potassium-6-bromo-2-naphthyl sulphate (Borden Co. Chemical division); postcoupling in both instances was with Fast Blue salt B (G.T. Gurr Ltd.).

(I) Esterase activity, usingα naphthyl acetate and

simultaneous coupling with Fast Blue B salt (I.C.I. Ltd.) (Pearse, 1960); and using the Indoxyl acetate method of Holt (1952).

(J) Steroid-3β-ol-dehydrogenase activity, with the method described by Wattenberg (1958). Incubations were conducted separately with 2 substrates, namely 17α hydroxy pregnenolone and pregnenolone, and lasted 2 hours.

(2) Ultrastructure. In preparation of this series the mice were killed at birth, 1,2,3,4,5,6, and 10 weeks. Neonatal testes were fixed intact in 1% buffered osmic acid (Zetterqvist,1956); teased preparations of the older gonads about 2 mm. in length were used. The material was dehydrated in methanol and embedded in araldite (Luft, 1961). Thin sections were cut on the Huxley and Porter-Blum microtomes, mounted on uncoated copper grids, and stained in 1% methanolic lead acetate. The preparations were screened in a Philips electron microscope EM.75B at 60 Kv with original magnifications of 1,100 to 11,000.

(3) Endocrine Activity. Androgen production by the testis may either be assessed directly by extraction and estimation of testicular androstenedione and testosterone, or indirectly by observations on androgen controlled characteristics. The direct method was not suited to the present project since it entails the use of 100 g. or so

of testicular tissue per estimation (the neonatal mouse testis weighs 2-3 mg.) and three indirect methods were accordingly chosen.

(A) Seminal Vesicle Weight. 80 animals were used in compiling the weight series, 10 being killed at each weekly interval between the end of the third and tenth weeks (inclusive) of post natal life. The vesicles were weighed together with their contained secretion in the unfixed state. The wet seminal vesicle tissue bulked from the 10 animals at each age group was divided into two parts; each part was weighed and the amount of fructose in one and citric acid in the other determined.

(B) Fructose estimation was conducted by the colorimetric method of Roe (1934).

(C) Citric acid estimation was carried out using the colorimetric method described by Speck Moulder & Evans in 1946, and modified by Lindner & Mann (1960).

In both of the above determinations a Hilger-Spekker absorptiometer was used.

To facilitate comparison of the three sets of results a quantitative analysis at each age group of the histochemical constituents of the testis and of the ultrastructural components of the Leydig cell was undertaken using the point method of Glagolev (1934) and Chayes (1949).

EXPLANATION OF PLATES

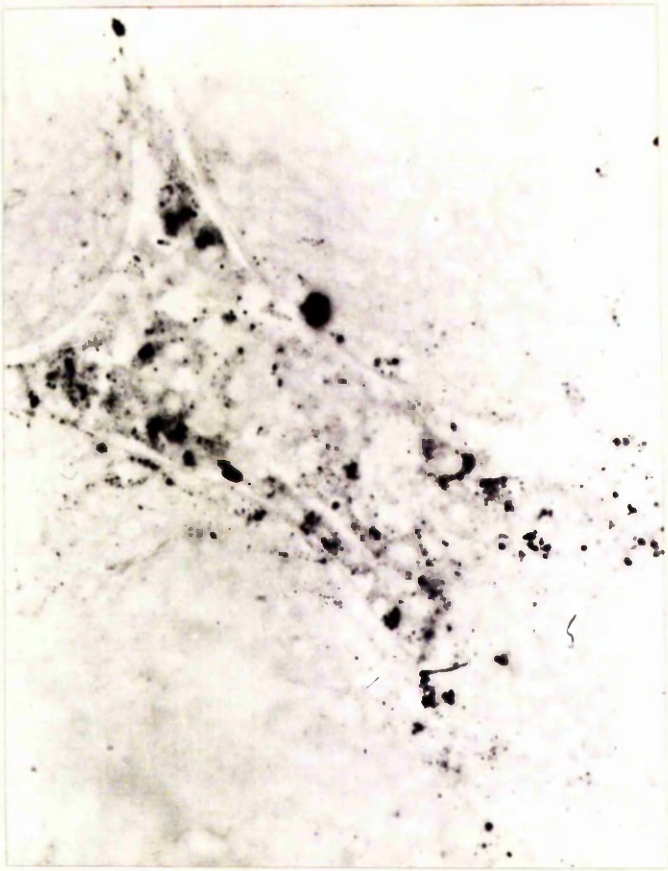
PLATE 1

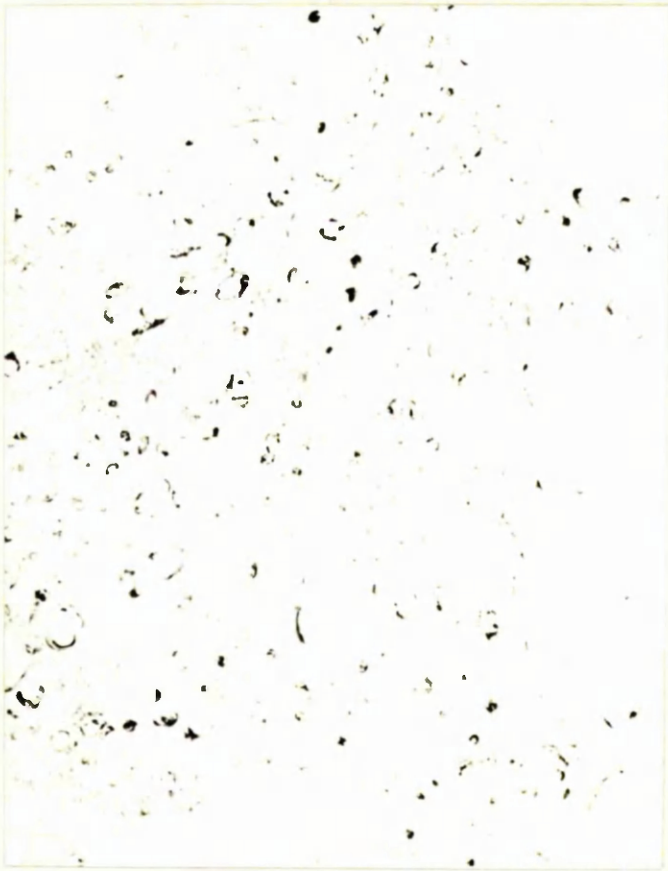
Fig. 1. Testis at 4 weeks, Ascorbic acid. Note the periphoral distribution of the silver granules. X 90.

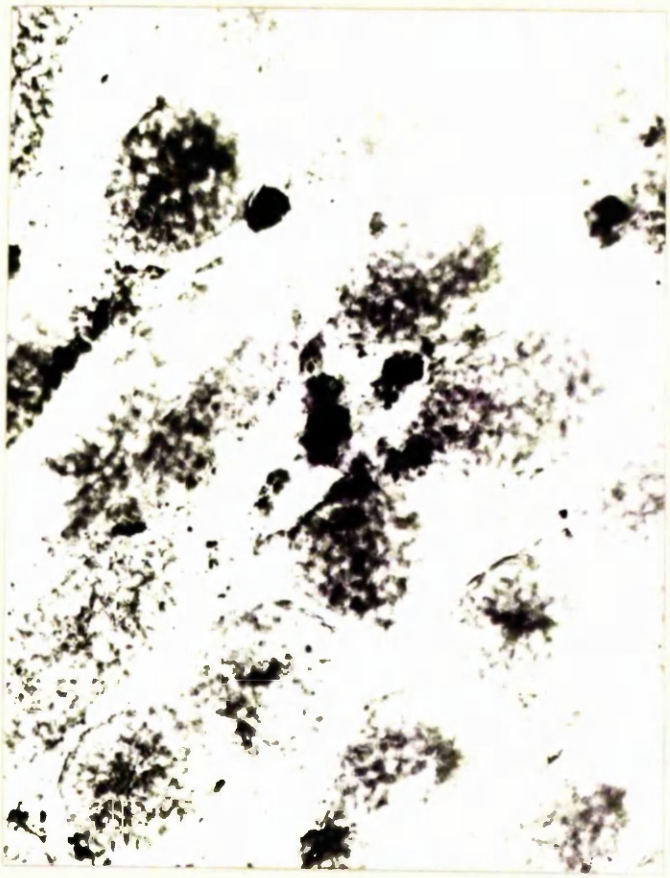
Fig. 2. Testis at 10 weeks. Note that the Ascorbic acid is much reduced in amount in the Leydig cells. X 90.

Fig. 3. Testis at 10 weeks. Note the small glucose particles adhering to the nuclei of the Leydig cells. X 360

Fig. 4. Testis at 2 weeks, Cholesterol reaction. Note that a few Leydig cells contain Cholesterol. X 90.







Histochemical sections at a magnification of 90 diameters were scanned with a modified grid (Hally, 1963) having points 1 cm. apart on two axes at right angles to one another, and the relative volume of Leydig tissue with a given histochemical component was derived as a percentage. In the same way electron micrographs of Leydig cells at low magnifications were scanned and the various cellular components expressed as a percentage of the cell volume.

RESULTS

(1) Histochemistry.

(A) Ascorbic Acid. At birth the Leydig cells of the mouse testis contain some minute black silver granules which denote the presence of ascorbic acid. These granules are distributed evenly through the cytoplasm. Thereafter the amount of ascorbic acid in the intertubular tissue seems to increase and the granules come to lie mainly in the peripheral cytoplasm (Pl. 1, Fig. 1). Between the eighth and tenth weeks ascorbic acid diminishes in amount to reach the characteristically low adult level (Pl. 1, fig. 2).

(B) Glucose is not present in the testis till the end of the tenth week, when minute amounts of silver appear in the Leydig cells in a juxtanuclear position (Pl. 1, fig. 3). Some of the particles are so close to the nuclear

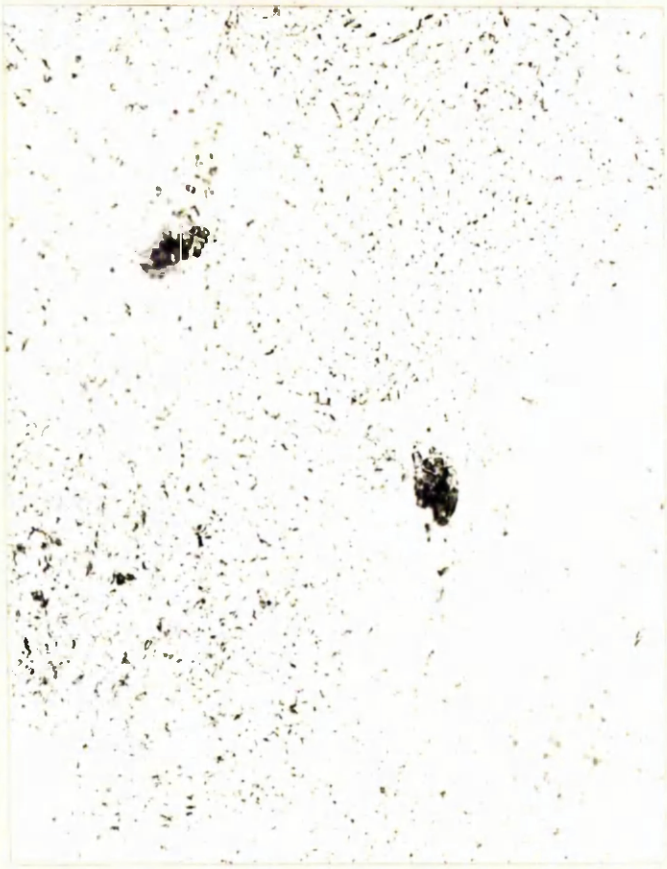
PLATE 2

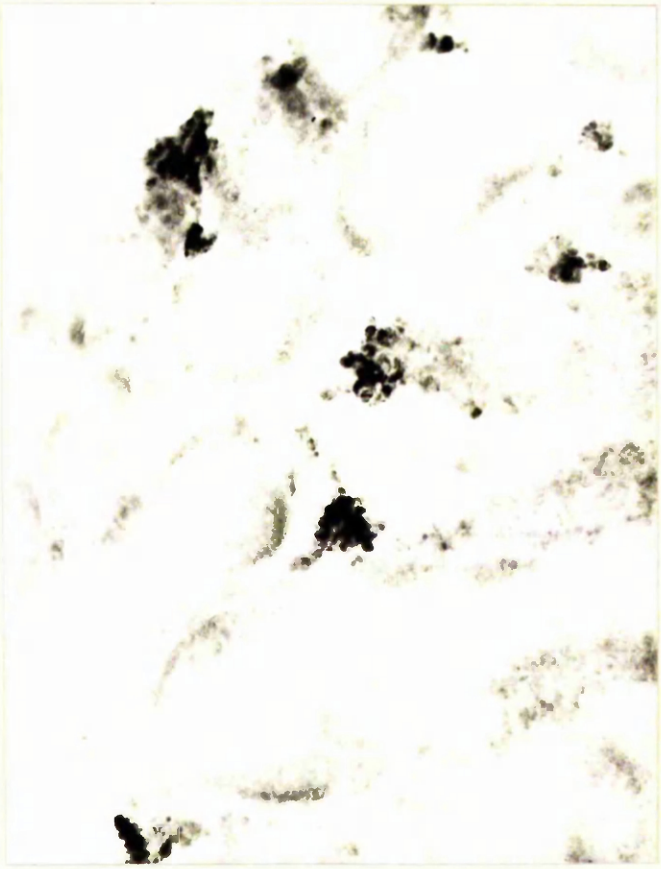
Fig. 5. Testis at 10 weeks, Cholesterol reaction. Note that practically all the Leydig cells contain Cholesterol. X 90.

Fig. 6. Testis at 8 weeks. Occasional Leydig cells contain lipofuscin pigment. X 90.

Fig. 7. Testis at 10 weeks. Note that some Leydig cells contain ~~α~~ ketols. X 90.

Fig. 8. Testis at 3 weeks, cytochrome oxidase. Note that a few Leydig cells contain this enzyme in large quantities. X 90.





membrane as to suggest that the glucose might be in some way attached to it.

(C) Cholesterol is absent from the neonatal and 1 week old testis. At the end of the second week a few interstitial cells contain cytoplasmic lipid droplets which give a deep green colour with Okamoto's reaction (Pl. 1, fig. 4). Cytological details tend to be obscured in such cells by the lipid masses. With advancing maturity more and more Leydig cells come to contain cholesterol until the adult interstitium is just a mass of Okamoto positive lipid (Pl. 2, fig. 5).

(D) Lipofuscin. Prior to the end of the fourth week of postnatal life no lipofuscin pigment is present in the Leydig cells; at that point a few cells possess some pigment giving a weakly positive ferric-ferricyanide reaction. The intensity of the staining and the number of Leydig cells involved increases with age. Discrete pigment granules are not present, rather the lipofuscin appears to be dispersed in the lipid masses throughout the cell cytoplasm (Pl. 2, fig. 6).

(E) α ketols, are wholly absent from the testis till the end of the tenth week, when a few of the Leydig cells are seen to have a number of tiny magenta-red coloured granules in their cytoplasm (Pl. 2, fig. 7). These red

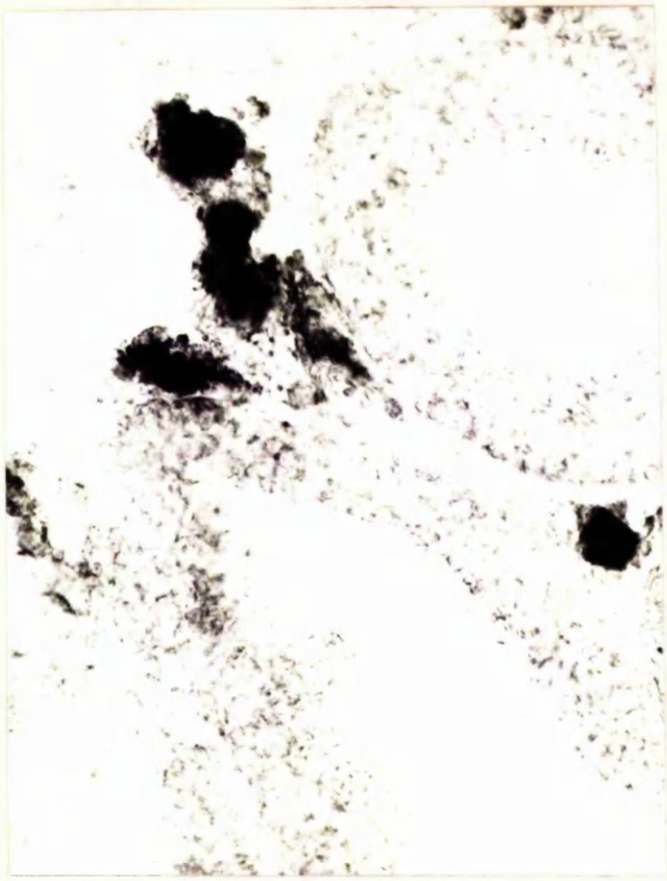
PLATE 3

Fig. 9. Testis at 10 weeks, cytochrome oxidase. Practically all the interstitium contain cytochrome oxidase. X 90.

Fig. 10. Testis at 3 weeks, succinic acid dehydrogenase. Note small formazan deposits in the Leydig cells. X 90.

Fig. 11. Testis at 10 weeks, succinic acid dehydrogenase. Note that the enzyme has increased considerably in quantity when compared with the three week picture. X 90.

Fig. 12. Testis at 10 weeks, aryl sulphatase. Note that the Leydig cells contain abundant aryl sulphatase activity. X 90.



granules are very much smaller than the typical lipid droplets found in Leydig cells at this age.

(F) Cytochrome Oxidase. No cytochrome oxidase activity could be demonstrated in the testes from neonatal, one week old, or two weeks old mice. In all older testes a proportion of the intertubular tissue exhibits enzymatic activity (Pl.2, fig. 8) and this proportion rises with age until most of the adult interstitium possesses cytochrome oxidase in histochemically demonstrable amounts (Pl. 3, fig. 9). The reaction product, indophenol blue, is widely dispersed in the Leydig lipids after even the briefest incubation with the result that the entire cytoplasm becomes a deep blue-violet colour. It is thus not possible to localise the site of enzymatic activity precisely in this tissue.

(G) Succinate dehydrogenase. This enzyme is not histochemically demonstrable until the end of the third week of postnatal life when a few interstitial cells have small blue diformazan deposits (Pl. 3, fig. 10). In older gonads progressively more Leydig cells give a positive reaction and the diformazan derived from the Nitro - B T is deposited in two sites; some appear in the form of discrete granules, as seen in the three week old testis, but the bulk of the diformazan is undoubtedly

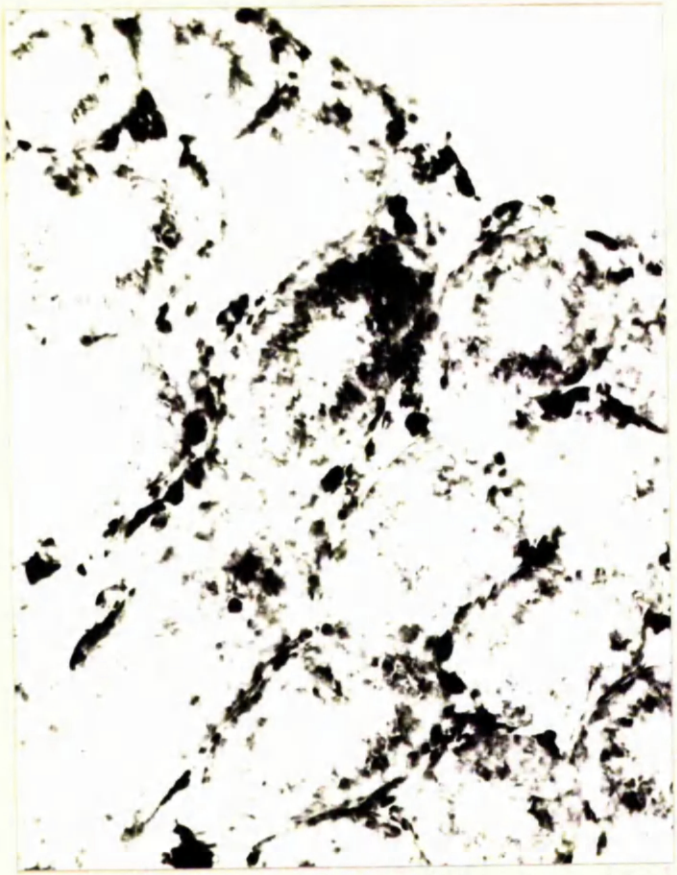
PLATE 4

Fig. 13. Testis at 14 days, α naphthyl acetate esterase: note that some of the Leydig cells are active. X 90.

Fig. 14. Testis at 4 weeks, α naphthyl acetate esterase: note that practically the entire interstitium is reactive. X 90.

Fig. 15. Neonatal testis. Note the fine metallic precipitate of indigo in most of the Leydig cells. X 90.

Fig. 16. Testis at 10 weeks, steroid-3-β ol dehydrogenase: 17α hydroxy pregnenolone. Note the marked reactivity of some Leydig cells when exposed to this substrate. X 90.



in solution in the Leydig lipids (Pl. 3, fig. 11). At any given age fewer Leydig cells possess succinate dehydrogenase activity than have cytochrome oxidase activity.

(H) Aryl sulphatase. Negative results were obtained with all age groups using potassium 6-benzoyl-2-naphthyl sulphate as a substrate. Incubation with 6-bromo-2-naphthyl sulphate for two hours indicated that aryl sulphatase activity was confined to the last (10 weeks) age group, when the interstitial cells are seen to contain myriads of minute pink staining droplets (Pl. 3, fig. 12). The pink colour is probably due to diffusion of the final product of the reaction in lipid, though it is noteworthy that the pink droplets are much smaller in size than the usual lipid droplets encountered at this age.

(I) Esterase. Using αnaphthyl acetate as a substrate, esterase activity is not demonstrable till the end of the second week of postnatal life, when some of the Leydig cells become quite strongly positive (Pl. 4, fig. 13). Over the next two weeks practically the whole interstitium comes to have an esterase capable of utilising this substrate (Pl. 4, fig. 14). This picture of intense activity is constant thereafter.

The distribution of esterase capable of acting on indoxyl acetate differs entirely from the foregoing esterase. It is present in the Leydig cells in fair amounts at birth (Pl. 4, fig.15) and dwindles steadily over the succeeding two weeks, to disappear entirely by the end of the third week. The blue precipitate of indigo is of an almost metallic appearance and is not soluble in the Leydig lipids to any appreciable extent.

(J) Steroid-3β-ol dehydrogenase. Using 17α hydroxy pregnenolone as the substrate, steroid-3β-ol dehydrogenase is found to be completely absent from the mouse Leydig cells up to and including the end of the ninth week of postnatal life. In the last group studied, some of the interstitial cells are clearly able to utilise this substrate, and there is no doubt that at ten weeks (Pl. 4, fig. 16) the Leydig cells utilise the substrate 17α hydroxy pregnenolone more readily than they use the second substrate pregnenolone.

The distribution of steroid-3-β ol dehydrogenase capable of acting on the substrate pregnenolone is quite different from that of the foregoing steroid-3-β ol dehydrogenase. Using pregnenolone as a substrate, practically the entire interstitium at birth is seen to possess intense steroid-3-β ol dehydrogenase activity,

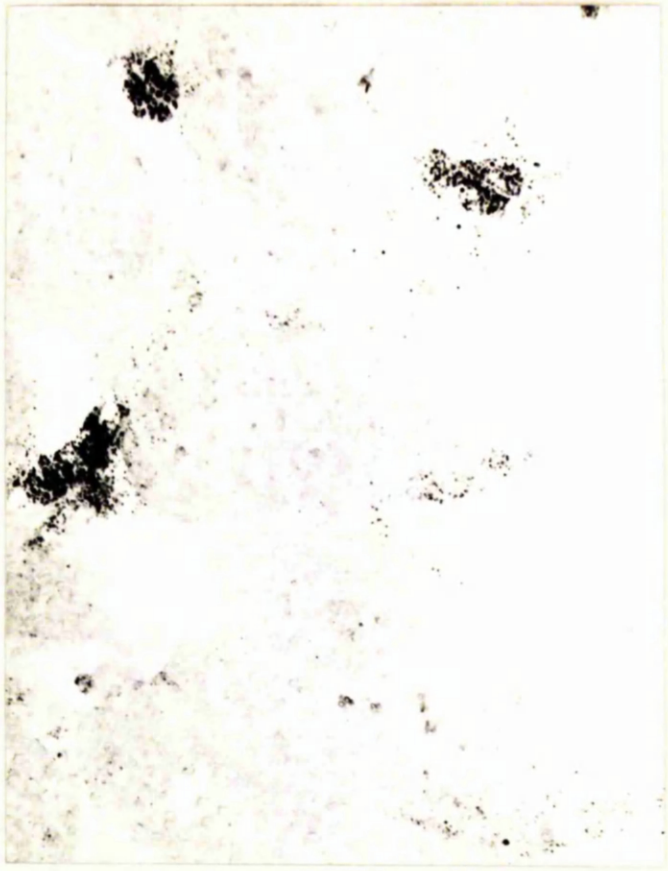
PLATE 5

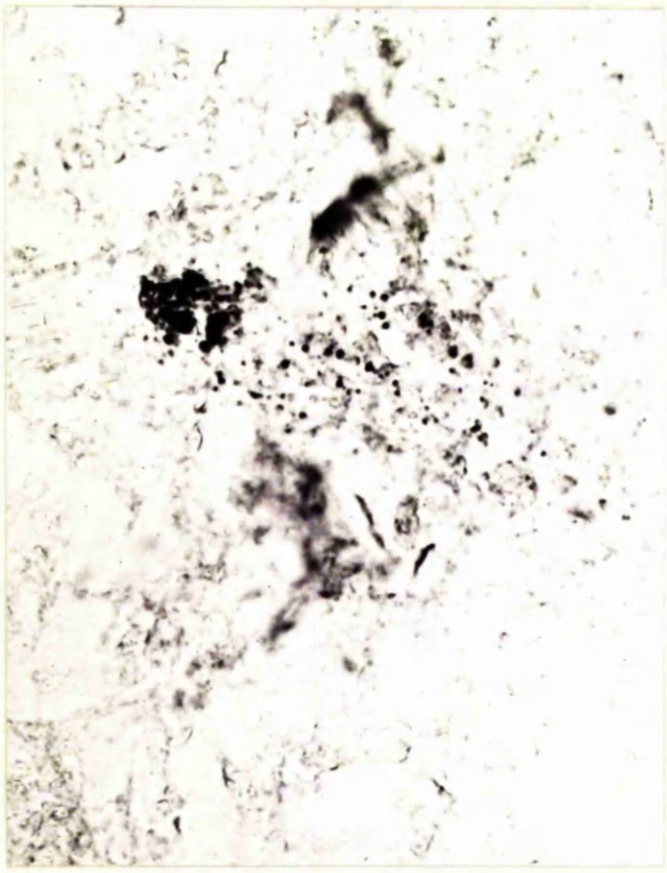
Fig. 17. Neonatal testis, steroid-3-β ol dehydrogenase. Note practically all the Leydig cells react with pregnenolone, at this age. X 90.

Fig. 18. Testis at 2 weeks, steroid-3-β ol dehydrogenase. Note that a number of Leydig cells still react strongly with pregnenolone at this age. X 90.

Fig. 19. Testis at 10 weeks, steroid-3-β ol dehydrogenase. Note the marked reduction in enzymatic activity with pregnenolone as substrate. X 90.

Fig. 20. Adult testis, steroid-3-β ol dehydrogenase. Note that Nitro B-T is deposited in the form of small crystals on the surface of the Leydig cell lipids. X 270.





(Pl. 5, fig. 17). During the ensuing two weeks the enzyme activity undergoes a marked reduction (Pl. 5, fig. 18) in amount and thereafter dwindles steadily to reach the stable and comparatively low adult level (Pl. 5, fig. 19). With both substrates the formazan derived from the Nitro B-T is deposited as small crystals on the surface of Leydig lipids (Pl. 5, fig. 20).

(2) Ultrastructure.

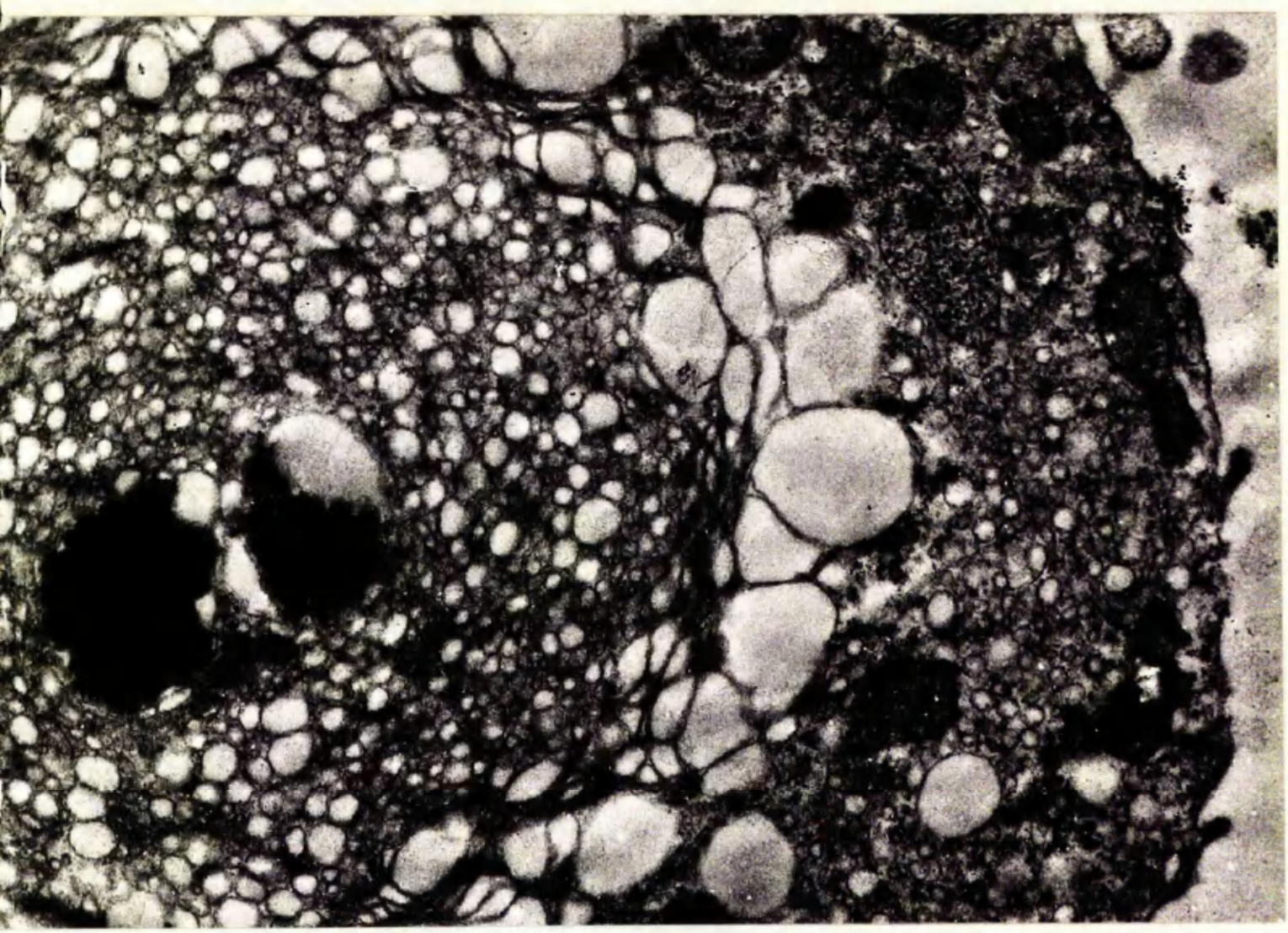
Two types of Leydig cell are seen in the interstitium of the growing mouse testis. By far the commonest are the large typically rounded or hexagonal cells occurring in groups of four to ten, and usually situated close to the capillaries, although a prominent extracellular space intervenes (Pl. 6, fig. 21). The plane cell membrane has small villous processes in a few cells (Pl. 6, fig. 22). Interdigitations between the membranes of adjacent cells are sometimes seen. Mitochondria are a prominent feature of these cells, accounting for a fair proportion of the cytoplasm. They are usually either rounded or oval in form, but elongated variants are occasionally visible. As a rule the matrix is of moderate electron density, and the numerous tubular cristae occupy most of the interior. Usually the cristae are simply infoldings of the inner

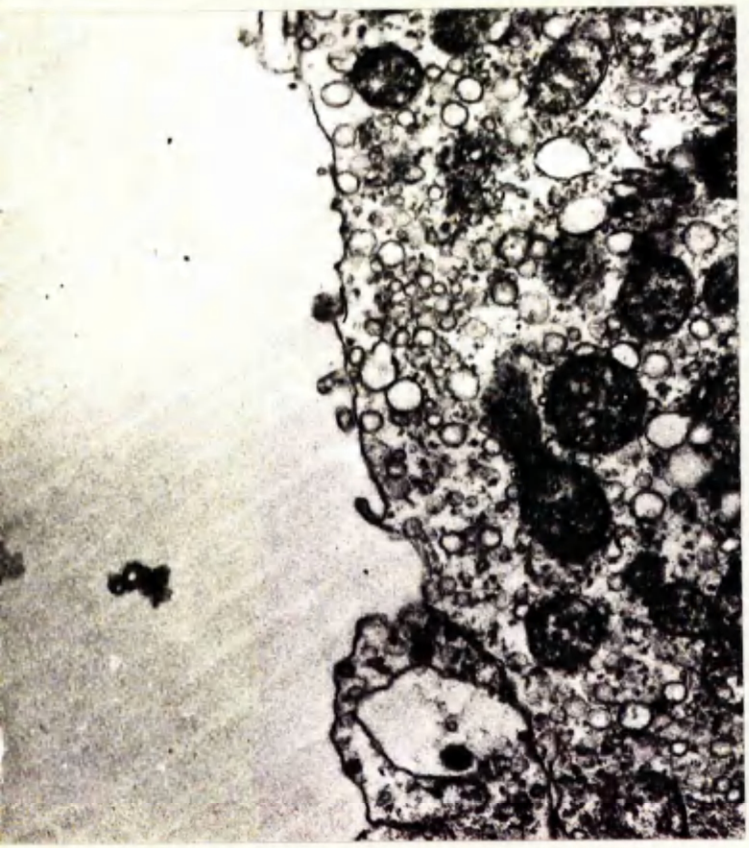
PLATE 6

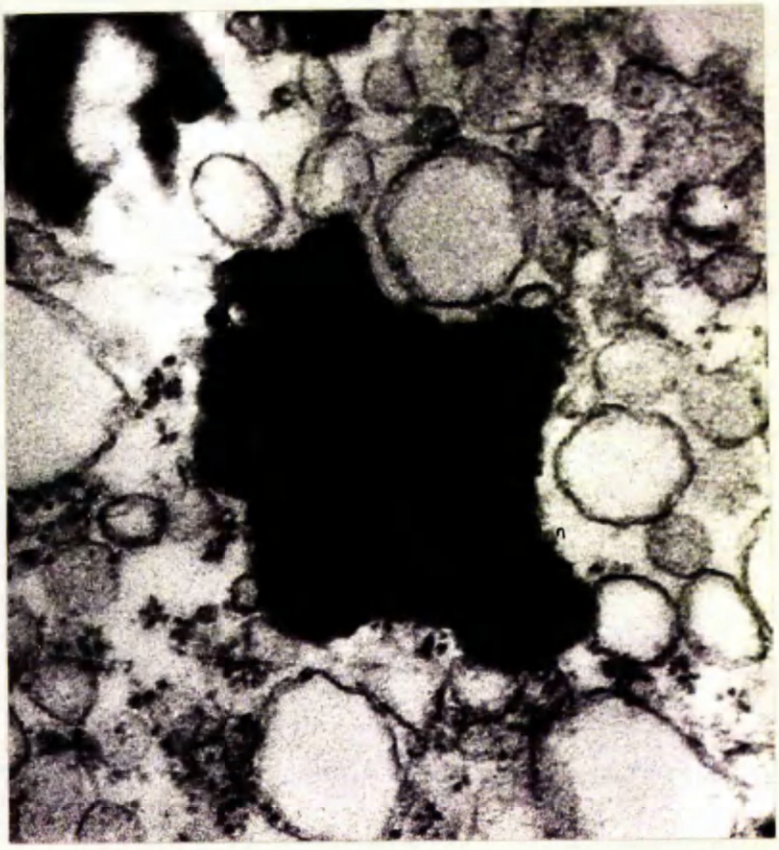
Fig. 21. Testis at 4 weeks. Electron micrograph showing Leydig cells with typical cytoplasmic constituents. X 27,000.

Fig. 22. Neonatal testis. Note the villus process projecting from the cell membrane of a 4 week old Leydig cell. X 12,000.

Fig. 23. Testis at 5 weeks. This electron micrograph shows a large irregular lipid inclusion with chatter marks due to section artefact. X 61,500.







limiting membrane, but more complex arrangements, including loops, are seen from time to time. Exceptionally, the inner membrane is split into two laminae over a portion of its length. Some mitochondria are two or three times larger than their neighbours; they have an empty, structureless centre and peripherally arranged cristae.

In a few cells grouped intercommunicating vesicles and paired membranes delimiting flat cavities were seen, but the Golgi apparatus was not usually prominent. The cytoplasm contains large numbers of predominantly small, smooth surfaced vesicles, though the size does vary. Most of these vesicles are apparently empty and are bounded by a single membrane which has, in some instances, a lining of moderately electron dense material. In the spaces between these vesicles numerous minute particles, probably ribonucleoprotein, occur in groups of twelve to twenty. These particles are not bound to the membranes of the small cytoplasmic vesicles described above.

The most striking cytoplasmic feature of the Leydig cells is the large irregular intensely osmiophilic lipid inclusions, usually measuring about 1 to 2 in diameter. In some cells these irregular lipid droplets may account for up to half the cytoplasm, but they may be entirely absent from others. Many of these lipids exhibit alternating

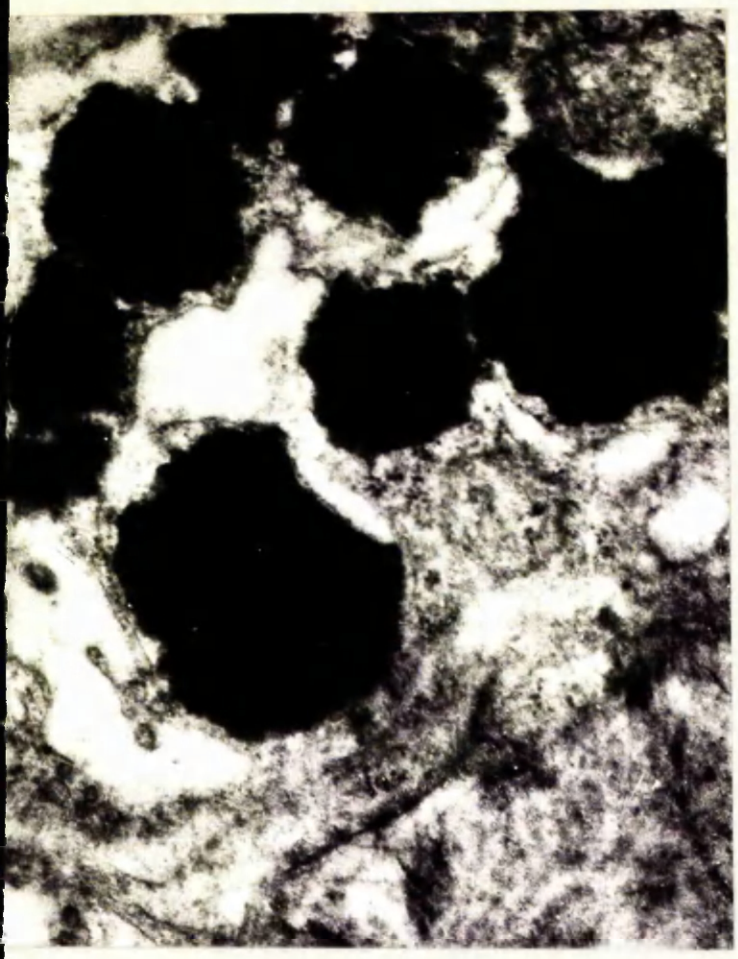
PLATE 7

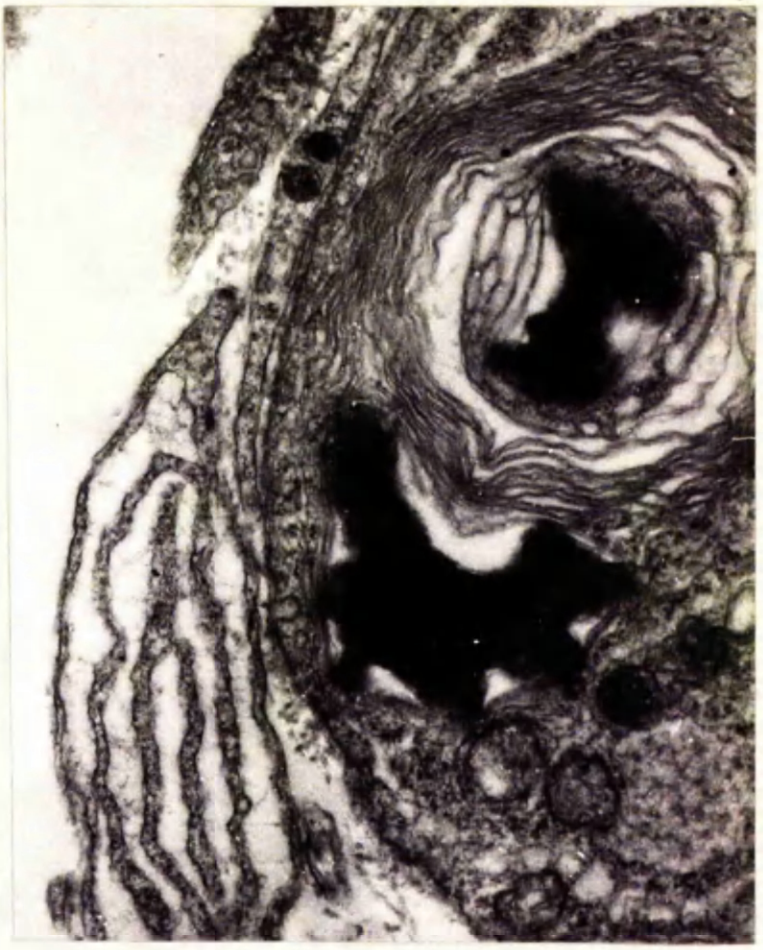
Fig. 24. Testis at 6 weeks. Note the large irregular lipid inclusions one has an incomplete membrane surrounding it. X 40,000.

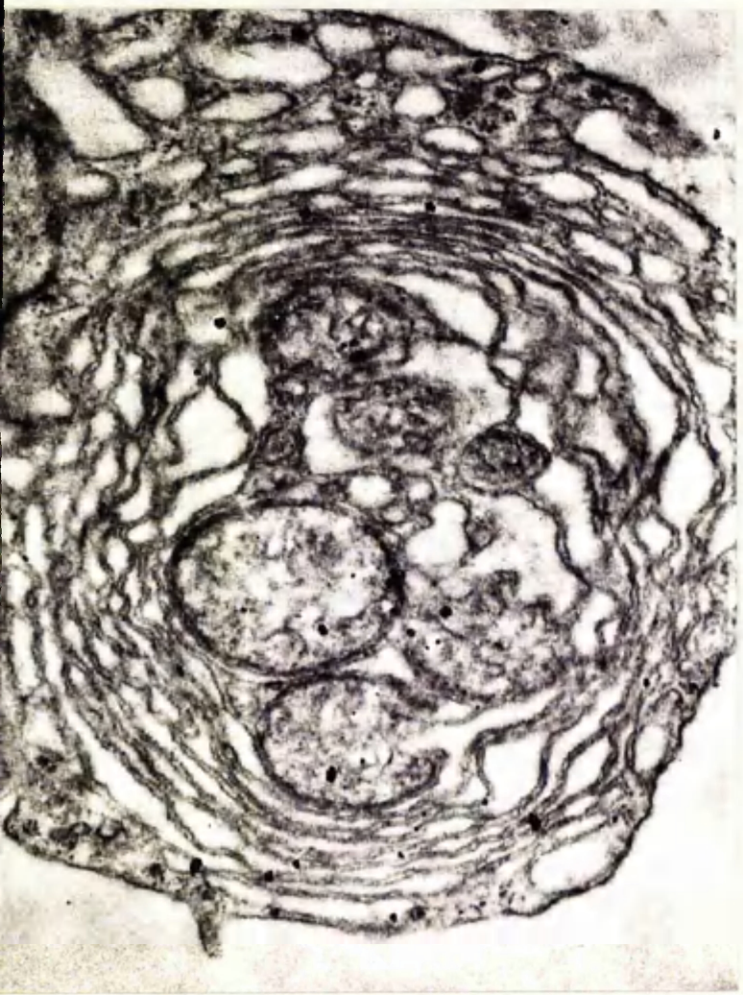
Fig. 25. Testis at 6 weeks. This micrograph shows two lipid inclusions surrounded by many concentric membranes. X 27,500.

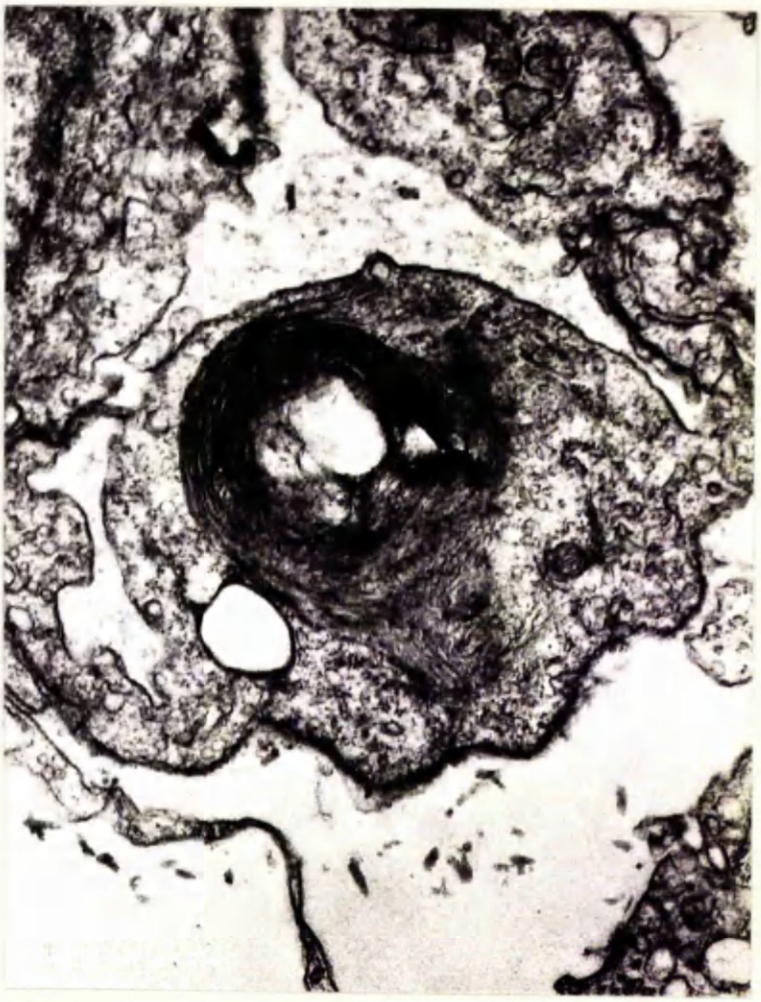
Fig. 26. Testis at 8 weeks. Instead of irregular lipid inclusions these membrane whorls occasionally contain structures akin to mitochondria. X 33,500.

Fig. 27. Testis at 4 weeks. Some membrane systems have an apparently empty interior. X 24,700.









light and dark bands (Pl. 6, fig. 23), doubtless section artefact.

A few of these irregular lipid inclusions are surrounded by a single or double membrane (Pl. 7, fig. 24), but the majority are not membrane limited. A third group, much rarer, of the irregular lipid masses is surrounded by many similar membranes (Pl. 7, fig. 25) which have interlacing connections, one with the other. Although it is difficult to be sure whether these are many concentric individual membranes or one spiral structure, the overall picture may be likened to an onion in cross section. These membrane whorls may contain an apparently typical mitochondrion in the interior as well as or instead of an irregular lipid inclusion (Pl. 7, fig. 26), or the interior may be empty (Pl. 7, fig. 27). Occasionally the irregular lipids bounded by a single membrane have an eccentric, apparently empty, area or vacuole contained within their limiting membrane (Pl. 8, fig. 28). Much smaller rounded lipid inclusions having no limiting membrane are fairly frequently seen, but they are never as common as the larger irregular lipid droplets (Pl. 8, fig. 29). Pleomorphic dense bodies (Pl. 8, fig. 30) usually rounded, are seen from time to time. Sometimes groups of parallel lamellae occur in the cytoplasm (Pl. 8, fig. 31). The lamellae are electron dense with a very fine, less dense,

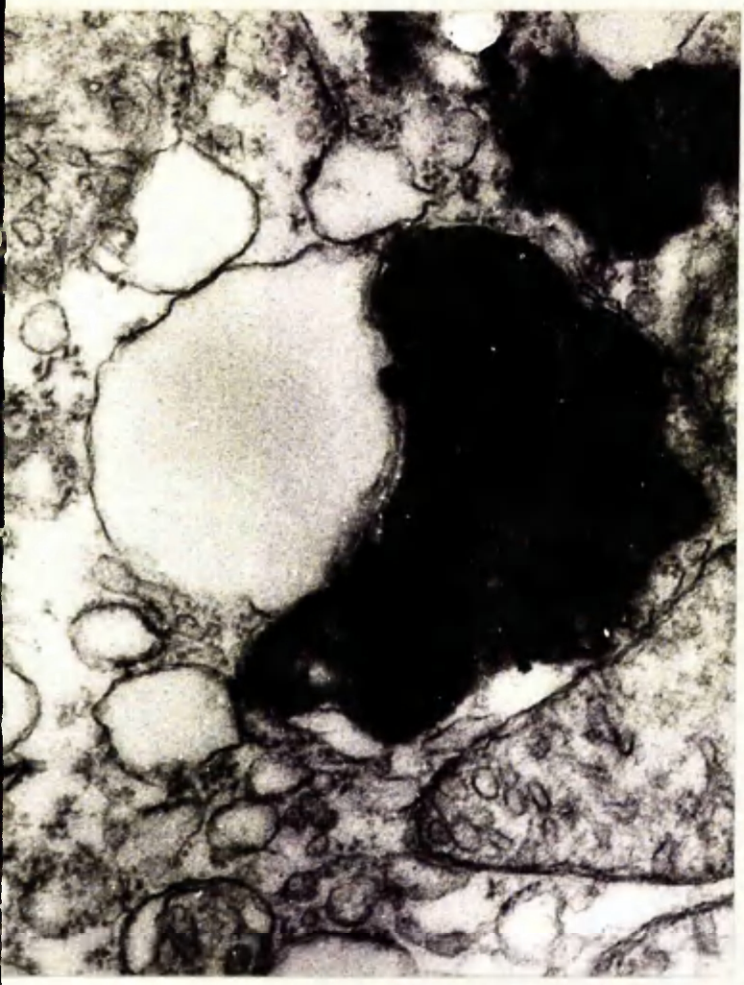
PLATE 8

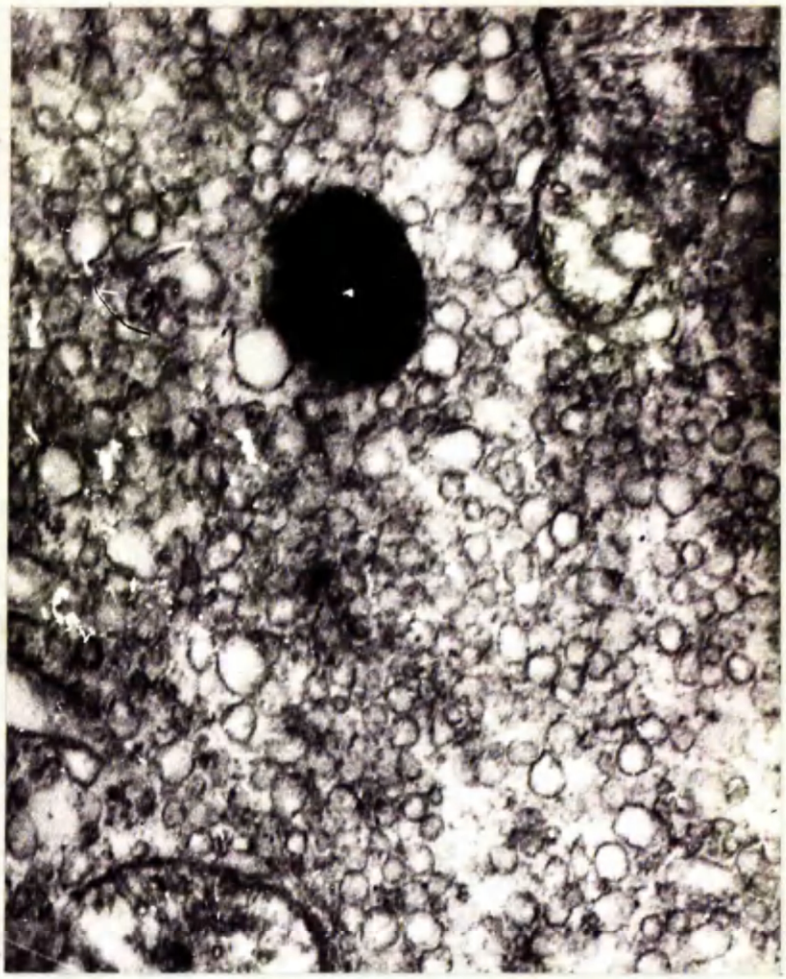
Fig. 28. Testis at 3 weeks. An irregular lipid inclusion showing a vesicle apparently contained in the same membrane. X 53,500.

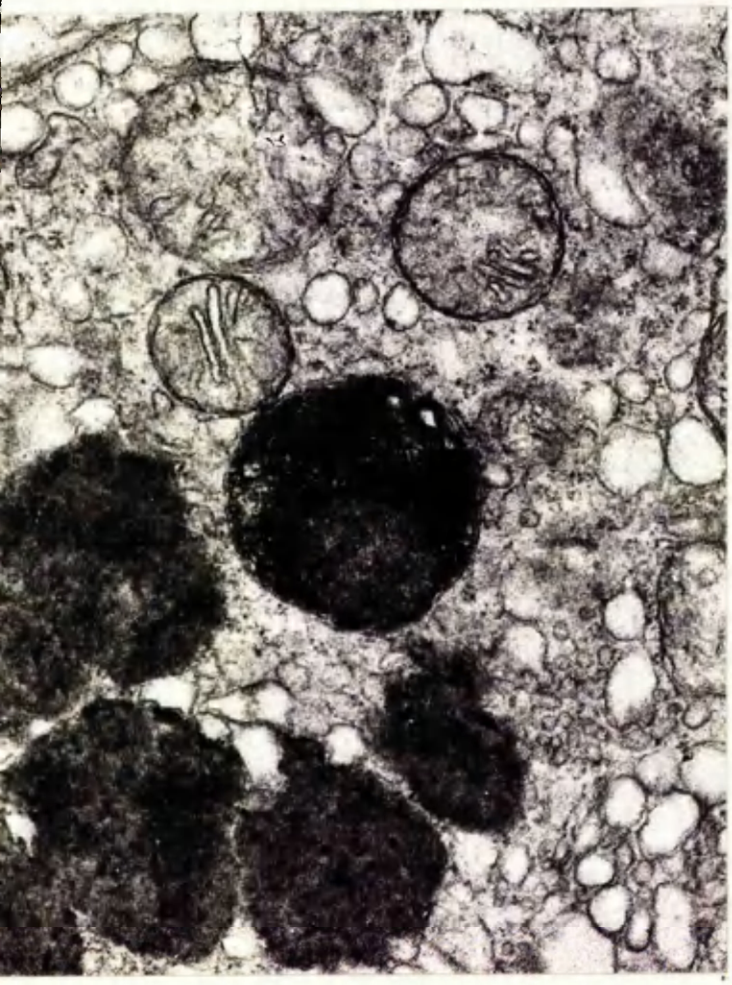
Fig. 29. Neonatal testis showing a small rounded lipid inclusion . X 38,500.

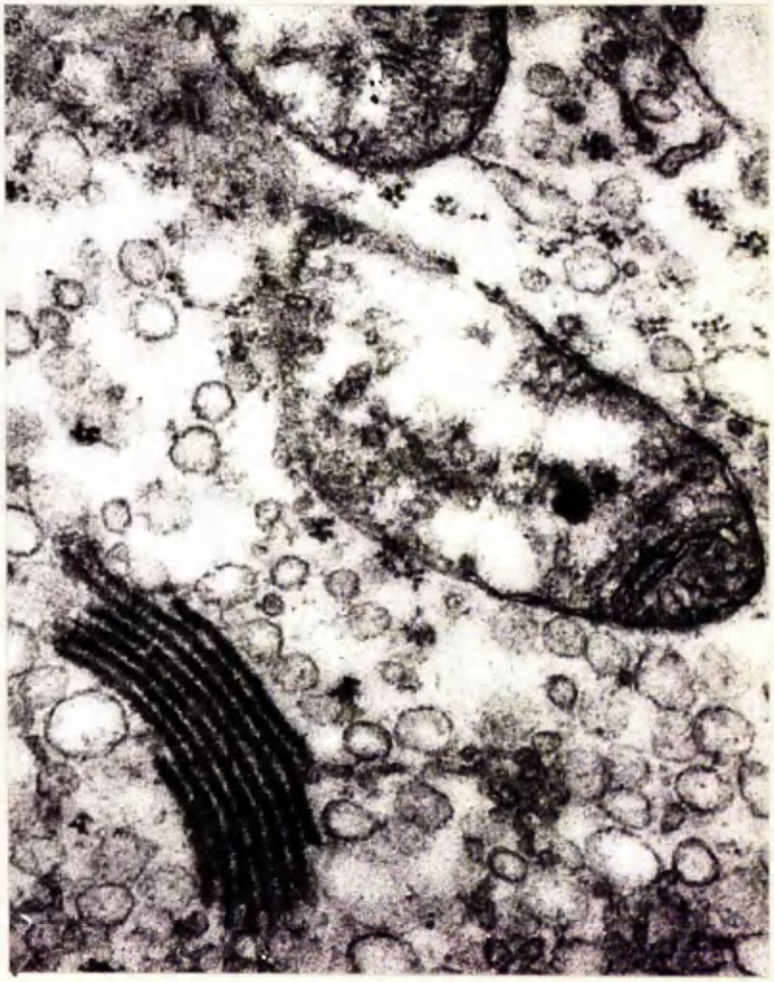
Fig. 30. Testis at 4 weeks showing a pleomorphic dense body (? Lysozome). X 36,000.

Fig. 31. Leydig cell aged 6 weeks showing parallel cytoplasmic lamellae. X 48,000.



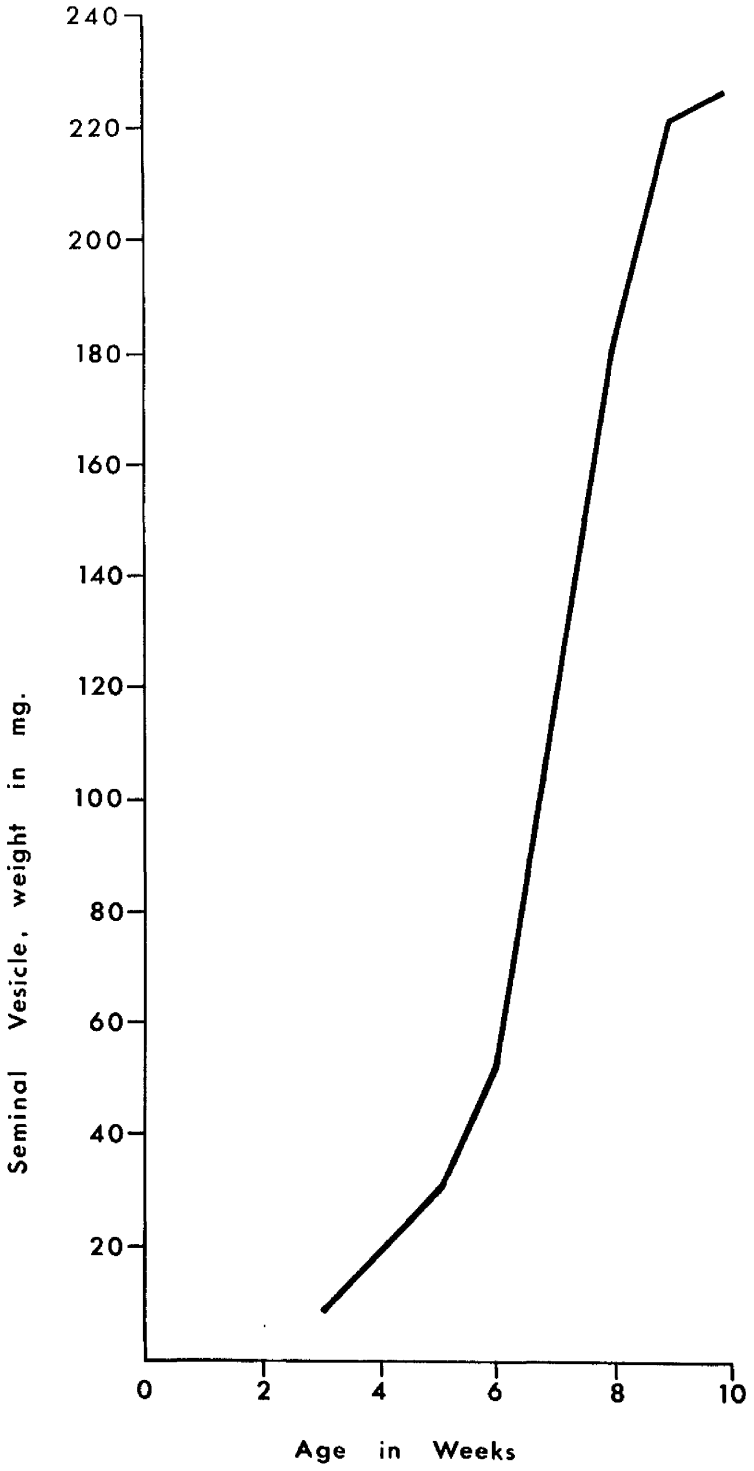






Text figure 1. The small vesicle growth curve is sigmoid in form and the bulk of the increase takes place between the sixth and eighth weeks of postnatal life.

Seminal Vesicle Growth Pattern



central strip, and the intervening areas between the lamellae present a slightly stippled appearance. The nucleus presents no features of note.

With the exception of the lamellated whorls, all the structures described above are present at all age groups; the irregular lipids, mitochondria, smooth reticulum, and lysozomes do not appear to change much quantitatively or qualitatively with age; the fine rounded lipid droplets diminish in number with advancing maturity; the membranous whorls are absent from the neonatal Leydig cell, fairly common between the second and sixth weeks, and rare at ten weeks.

The second type of Leydig cell noted is a spindle shaped cell, usually seen near the seminiferous tubules and having most of the features of the typical Leydig cell excepting the membrane whorls. The cells appear to be intermediate in type between fibroblasts and typical Leydig cells, and are to be found in testes of all age groups.

(3) Endocrine Activity.

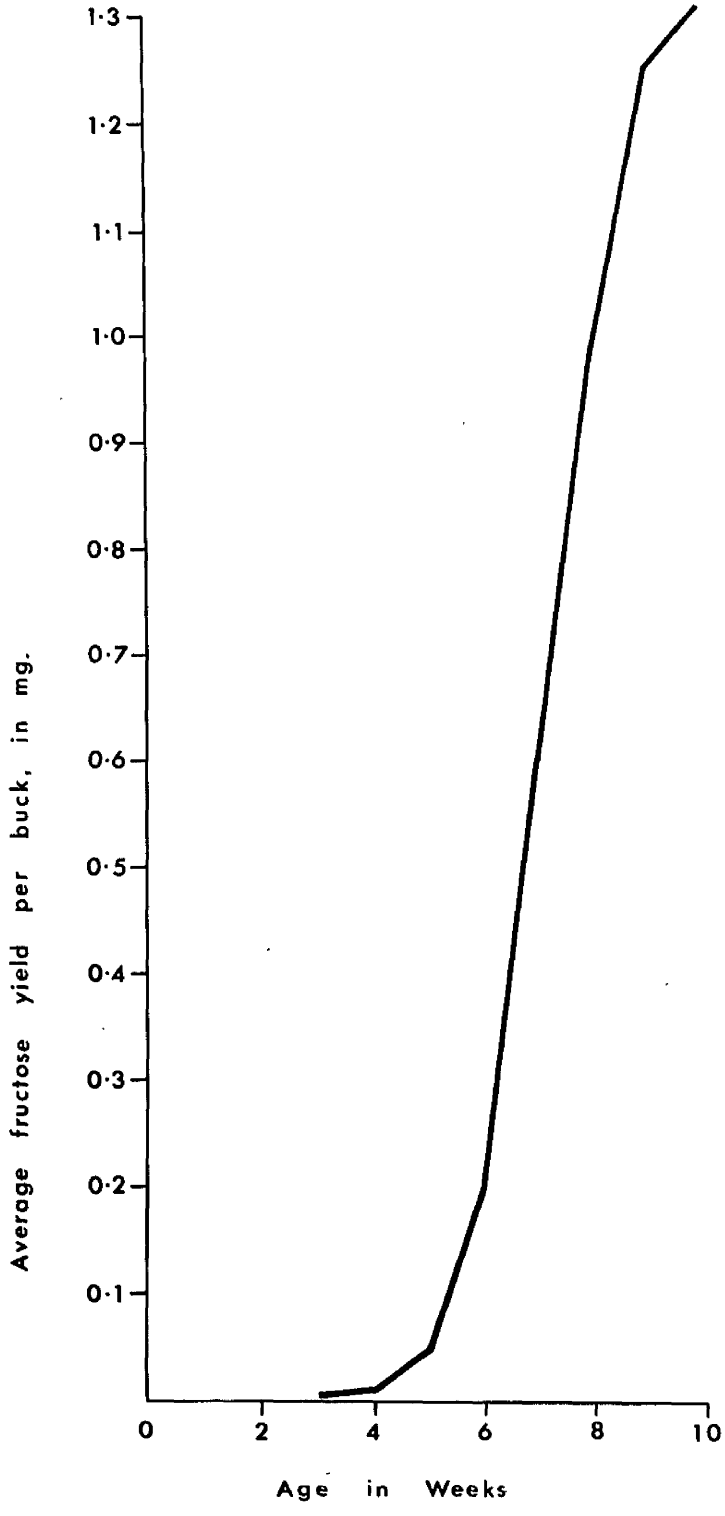
(A) Seminal vesicle weight. The salient features are summarised in Table I and in the sigmoid growth curve curve shown in Text Fig. 1. The vesicles are not large enough for weighing until the end of the third week;

Age Series.

Age in weeks	0	1	2	3	4	5	6	7	8	9	10
Ave. Sem. Ves. Wt.	-	-	-	8.3	20	31	53.2	117.5	180	220	225 mg.
Fructose. Yield per buck	-	-	-	0.003	0.01	0.05	0.2	0.6	0.99	1.25	1.31 mg.
Concentration per 100 g. Sem. Ves.	-	-	-	35	82	200	397	512	645	667	675 mg.
Citric Acid. Yield per buck	-	-	-	-	-	0.01	0.04	0.13	0.3	0.44	0.47 mg.
Concentration per 100 g. Sem. Ves.	-	-	-	-	-	32	75	111	166	200	208 mg.
Ratio:- of concentration $\frac{\text{Fructose}}{\text{Citric Acid}}$	-	-	-	-	-	$\frac{6.6}{1}$	$\frac{5.3}{1}$	$\frac{4.6}{1}$	$\frac{3.9}{1}$	$\frac{3.3}{1}$	$\frac{3.2}{1}$

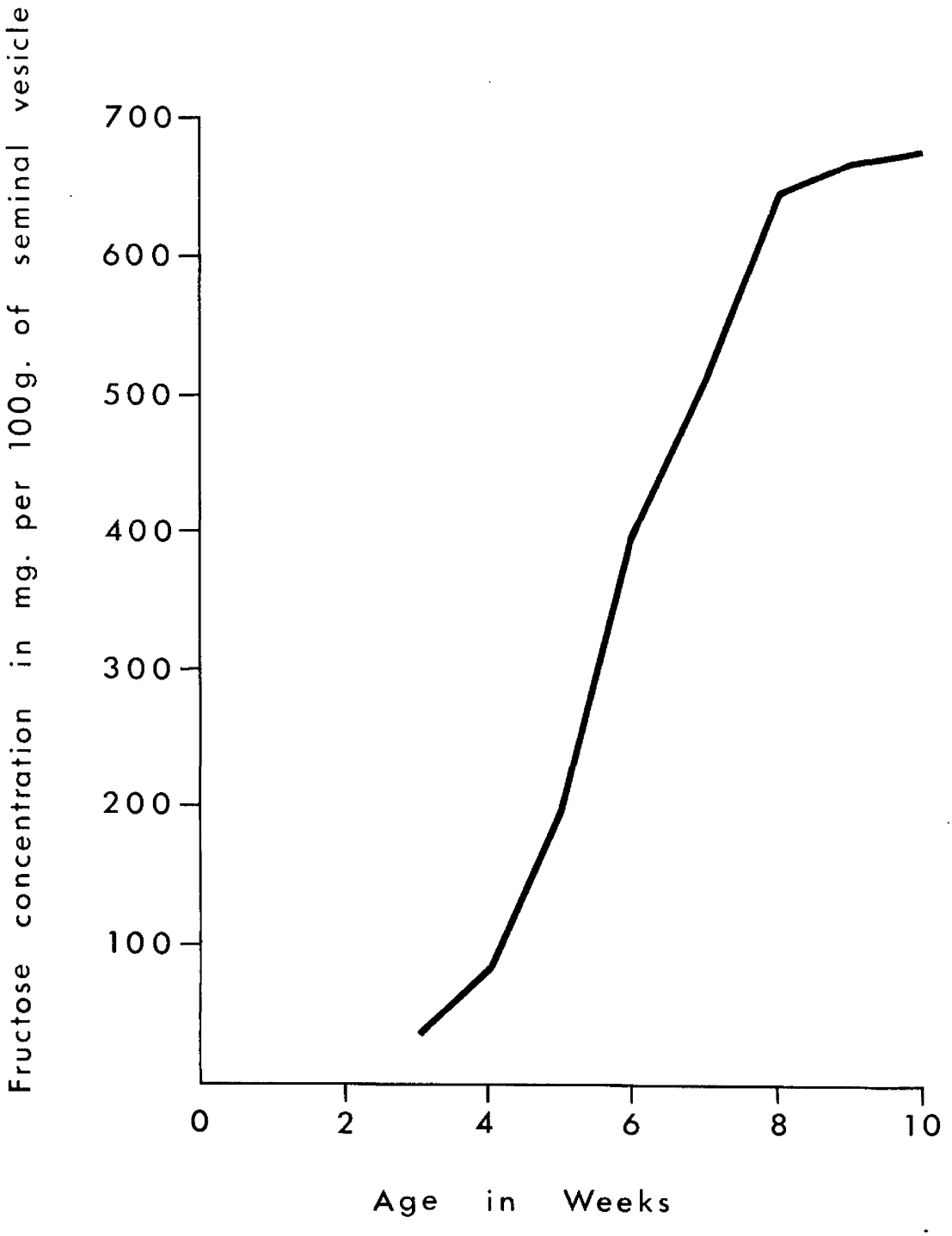
Text figure 2. The Fructose yield per buck gives a sigmoid growth curve with maximal growth taking place between the 6th and 8th weeks of life.

Fructose Yield



Text figure 3. The fructose concentration gives the familiar sigmoid growth curve.

Fructose Concentration



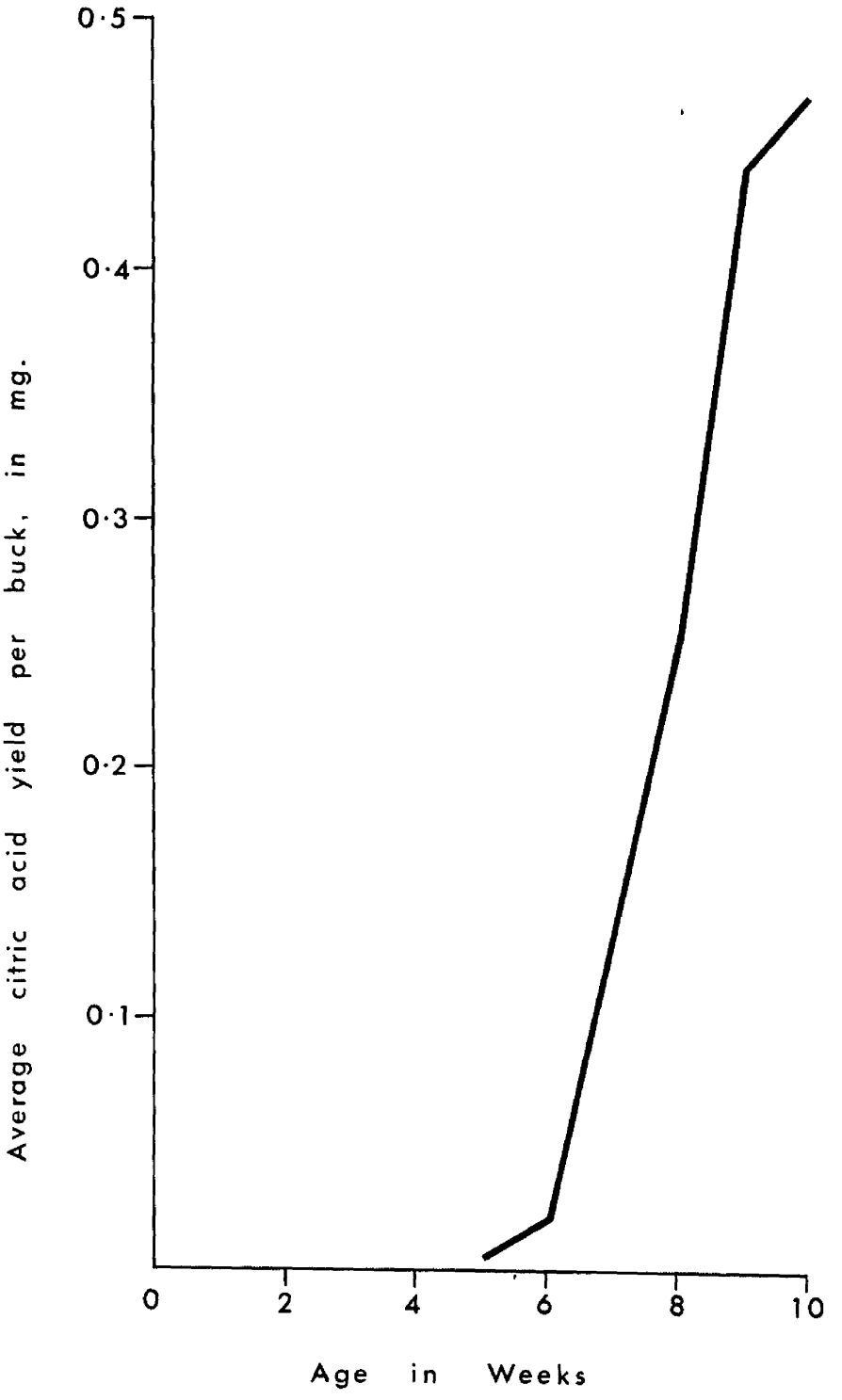
growth thereafter is largely completed by the end of the eighth week of postnatal life.

(B) Fructose. This sugar is present in the seminal vesicles of the mouse at all age groups covered by the present series. The yield per buck (Table I, Text Fig. 2) rises from 0.003 mg. at three weeks to 1.31 mg. at ten weeks (Pl. 9, fig. 32). These figures are clearly the combined result of two factors, namely, the growth of the seminal vesicles, and the rising concentration of fructose per unit weight with age. The fructose concentration (Table I, Text Fig. 3) rises from 35 mg. per 100 g. at three weeks to 675 mg. per 100 g. at ten weeks. The bulk of the rise in concentration is completed by the end of the eighth week of extra-uterine life, and the growth curve is sigmoid in form.

(C) Citric Acid. No citric acid can be detected in the seminal vesicles till the end of thefifth week of extra-uterine life (Table I). Thereafter the yield per buck rises from 0.01 mg. to 0.47 mg. (Text Fig. 4) (Pl. 9, fig. 33). As with the fructose results, the increasing yield of citrate with age per buck represents a combination of seminal vesicle growth with a rising concentration of citrate with age. The citric acid concentration rises from 32 mg. per 100 g. of seminal

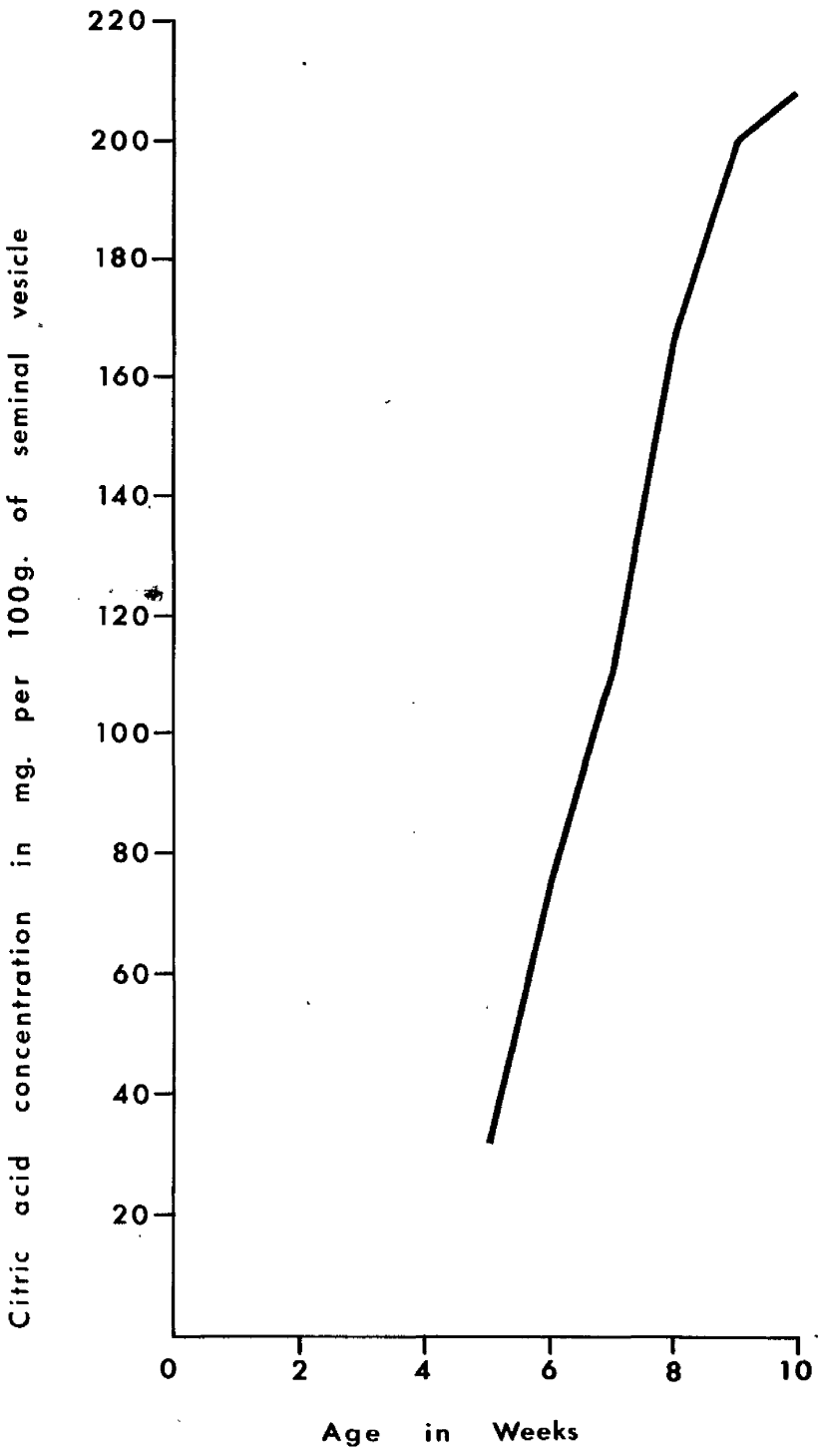
Text figure 4. The Citric Acid yield per buck gives an "S"-shaped growth curve with the maximal increase occurring between the 6th and 8th weeks of life.

Citric Acid Yield



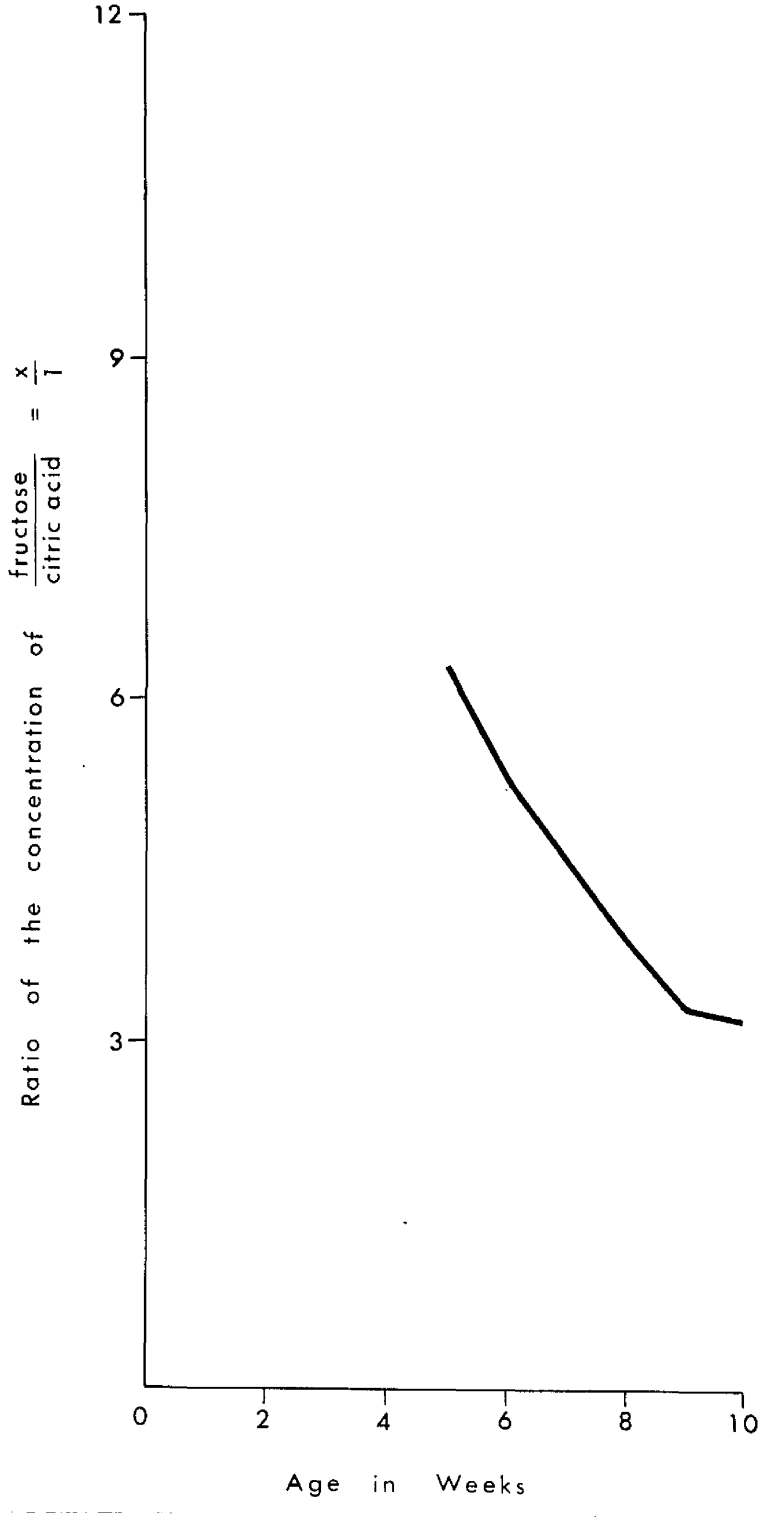
Text figure 5. The Citric Acid concentration resembles the Citric Acid yield expected with the exception that the initial part of the "S" curve is missing for technical reasons: it is not possible to assay Citric Acid concentration below 20 mg. per 100 gms. of tissue accurately.

Citric Acid Concentration



Text figure 6. The ratio of Fructose to Citric Acid is seen to fall steadily with age between the 5th and 10th weeks of life.

Ratio of $\frac{\text{Concentration of Fructose}}{\text{Concentration of Citric Acid}}$



vesicle at five weeks (Table I, Text Fig. 5) to 208 mg. per 100 g. at ten weeks. Again, the largest part of the rise in concentration takes place by the end of the eighth week, and again the growth curve is sigmoid in form.

The ratio of the concentration of fructose per 100 g. of seminal vesicle to citric acid per 100 g. was also calculated for each age group once both constituents were present, and the results are summarised in Table I. Text Fig. 6 indicates that the ratio of fructose to citric acid falls steadily from 6.6 to 1 at 5 weeks to the adult level of 3.2 to 1 at 10 weeks.

(4) Quantitative Results.

The quantitative histochemical data are summarised in Table 2. The ascorbic acid preparations were poorly stained centrally, apparently as a result of the silver penetrating the block more slowly than the buffer front which dissolves out the readily soluble vitamin C. While a peripheral rim in each block was well stained, it was felt to be too small to permit quantitative analysis, ( ).

Intertubular tissue contains no glucose till the tenth week when interstitial tissue having glucose demonstrable represents 0.6% of the testis. Intertubular

tissue containing cholesterol rises from 1.5% of the testis at 2 weeks, when it is first present, to 5.3% of the testis at 10 weeks. Lipofuscin is absent till the fourth week and then increases progressively from 0.3% to 1.4%. ∝ ketols are not demonstrable till the end of the tenth week when the Schiff stainable intertubular tissue represents 0.6% of the testis. Intertubular tissue having cytochrome oxidase and succinic dehydrogenase activity accounts for 2.2% and 0.6% respectively of the testis at three weeks, when both enzymes first appear; thereafter the proportion of reactive intertubular tissue rises steadily, and the corresponding ten week figures are 4.6% and 1.2% respectively.

Aryl sulphatase is absent till the end of the tenth week of postnatal life (Table 2). ∝ naphthyl acetate esterase and indoxyl acetate esterase have specific and different quantitative age distributions, and these are summarised in Table 2 as is the quantitative distribution of the steroid-3-β-ol dehydrogenases acting on 17 hydroxy pregnenolone and pregnenolone. The quantitative ultrastructural results are summarised in Table 3.

DISCUSSION

The interstitial cells of the testis, in common with steroid producing cells in other sites, e.g. adrenal

TABLE 3

Age in weeks	Mitochondria as % of cytoplasm	Irregular Lipids as % of cytoplasm	Rounded Lipids as % of cytoplasm
0	12.1%	6.0%	0.5%
1	12.2%	5.7%	0.3%
2	13.3%	5.3%	0.2%
3	12.5%	6.9%	0.3%
4	12.6%	7.1%	0.1%
5	14.0%	5.6%	0.1%
6	12.0%	6.8%	0.2%
10	14.1%	5.4%	0.1%

cortex (Svirbehj & Szent-Gyorgi, 1933) contain granules which reduce silver nitrate. In the neonatal testis, the even distribution of the granules through the Leydig cytoplasm resembles the distribution of mitochondria demonstrated in the electron micrographs, and accords with the observations of Giroud (1938) who noted a close correlation between the size and distribution of the silver stained granules and that of the mitochondria. In the Leydig cells of more mature animals, however, the peripheral distribution of the silver granules does not correspond with the mitochondrial disposition disclosed in electron micrographs, and these latter observations do not conform with Bourne's (1944, 1950) findings that in the pituitary and adrenal the distribution of silver stained granules is identical with that of mitochondria stained with Janus green. Nor do the present observations support the views of Barnett & Fisher (1943) that precipitation of silver nitrate depends on the presence of various interfaces, since the electron micrographs show quite clearly that interfaces are not peculiar to, or are predominantly found in the peripheral cytoplasm. In view of the lack of correlation between the sites of silver precipitation in the older interstitium and the ultrastructure of the Leydig cell, it seems probable that if Vitamin C has a mitochondrial location then the pH

conditions prevailing in the impregnations produce severe intracellular damage in the Leydig cells. There appears to be no satisfactory solution to the problem of ensuring even block penetration with silver at present (Ohnacker, 1960).

Muller (1955) found glucose to be present in fair quantities in the mouse liver, but absent from the kidney: glucose does not appear to have been noted in the testis previously, but it is constantly present in minute amounts in the Leydig cell of the adult mouse. The silver particles are finer, longer, and have an entirely different cellular localisation from those associated with the presence of ascorbic acid. Their apparent close association with the nuclear membrane is of unknown significance. In this connection it is interesting to note that Niemi and Ikonen (1962) found a glucose-6-phosphate dehydrogenase in the Leydig cells of the rat.

Cholesterol is known to be present in the Leydig cells of the birds Sturnus vulgaris, Musciapa parva, Regulus regulus, Acrocephalus schoebaenus, and various members of the groups Turdus and Sylvia (Lofts & Marshall, 1957); it shows a seasonal variation in these animals, being usually absent during the quiescent phase and increasing towards the onset of the breeding season.

This avian relationship between Leydig cholesterol and sexual activity closely resembles the situation in the growing mouse where the immature sexually inactive animal has no Leydig cell cholesterol, while the fertile adult possesses a cholesterol laden interstitium. Like the mouse, human adult interstitial cells contain cholesterol (Mancini et al., 1952).

In common with the interstitial cells of the urodelan amphibian (Humphrey, 1921), of the rooster (Loisel, 1904), and of the woodchuck (Rasmussen, 1932) at the close of the breeding season, and of the adult human (Fawcett & Burgos, 1960), some of the Leydig cells of the mature and nearly mature mice contain lipochrome pigment or lipofuscin. The dispersion of the lipochrome pigment through the lipid masses in the present material is in accord with the views of Pearse (1960) who feels that the lipofuscin propigment is derived from a lipid precursor, becomes P.A.S. positive, and is progressively oxidised to a yellow-brown pigment: during this maturation the lipofuscin becomes steadily less soluble in acetone and less sudanophilic. The P.A.S. positive diastase-resistant granules previously described in the Leydig cell cytoplasm of the growing and adult mouse (Baillie, 1961) may possibly be related to lipofuscin synthesis. Gomori (1955) and Gedgik & Bontke (1956) have noted the presence

of a non-specific esterase capable of hydrolysing Naphthol AS acetate associated with various lipofuscins; Pearse has noted esterase activity using indoxyl acetate as a substrate in nerve cells containing lipofuscin pigments. In this laboratory (unpublished observations) the age distribution of Naphthol AS acetate esterase has been found to be identical with that of the lipofuscin, while esterases demonstrated by other substrates (e.g. Naphthol AS, LC acetate, and naphthol AS. D. acetate) show no obvious relation to lipofuscin formation. The wide dispersion of lipochrome pigment in the mouse Leydig cell makes it difficult to be sure whether lipofuscin and Naphthol AS acetate esterase have the same cytological location, but their identical age distribution favours Pearse's (1960) view that they are constantly associated, rather than that of Gomori (1955) who doubted the accuracy of localisation of the enzyme in the pigment.

α ketols are not present in the Leydig cell of the mouse testis till the animal is adult, and in this respect their age distribution resembles that of glucose, aryl sulphatase, and 17-hydroxy progesterone-3-Bol dehydrogenase. Having regard to the known association of the last two tissue components with steroid synthesis, the writer is satisfied that tissue α ketols are demonstrable by the method of Pearse (1960) notwithstanding the fact that

the ketosteroid content of tissues is minute (Olson et al., 1944; Griffiths, personal communication; Rogers & Williams, 1947). While the overall concentration of steroid per tissue slice is minute, the positive reaction given by a few cells to this test is probably explained on the basis that steroid hormones are restricted to a few cells and the amount in one or two is high enough to give a positive histochemical reaction.

Cytochrome oxidase activity has been described in the Leydig cells of foetal, prepubertal, and adult mice of the BALB/c Jax mice (Hitzeman, 1962), becoming more strongly positive with age. Lack of histochemically demonstrable cytochrome oxidase before the end of the third week of postnatal life in the Leydig cells of the mice used in the present study is probably due to a strain difference: its increase thereafter in reaction intensity accords with Hitzeman's findings, and probably reflects a rising cytochrome oxidase complement per mitochondrion, since the mitochondrial content of the Leydig cell does not increase significantly with age (Table 3).

The age distribution of succinic dehydrogenase in the present material closely resembles that of cytochrome oxidase, although at any given age the latter is histochemically more strongly positive. The relative ease of demonstrating succinate dehydrogenase in the Swiss

white strain contrasts with Hitzeman's equivocal findings in BALB/c Jax mice and further underlines the strain differences between the two groups. The steady increase in succinic dehydrogenase activity with age resembles that of cytochrome oxidase, and both are probably related to increasing energy requirements in connection with steroid synthesis. As with cytochrome oxidase, the succinic dehydrogenase increase with age is probably the result of a rising mitochondrial concentration since these organelles, as already noted, do not increase with age.

At least three aryl sulphatase enzymes have been described (Roy, 1956a) and it seems (Roy, 1956b) that species lacking aryl sulphatase C are unable to conjugate steroids with sulphate groupings. Sulphatase C is thus thought (Roy, 1956b) to act as a steroid sulphatase, and it has been shown to be capable of synthesizing dehydroepiandrosterone sulphate. It is interesting to note that the appearance of this enzyme coincides with that of the 17 hydroxy progesterone-3-β-ol dehydrogenase, and it is tempting to implicate it in the adult route of synthesis of androgen. The cytoplasmic location of this enzyme is unsure, since the final reaction product is diffused as minute pink droplets throughout the Leydig cytoplasm, but the droplets do not appear to correspond with Leydig

lipids, either in size or in distribution. This leaves the mitochondria and endoplasmic reticulum as the likely "anchorage" of aryl sulphatase, and it is significant that the latter has been particularly thought to play a part in steroid synthesis (Fawcett and Burgos, 1960).

The distribution of naphthyl acetate esterase (aliesterase - Pease, 1960) in the present mouse strain corresponds with Hitzeman's (1962) experience in BALB/c Jax mice, with the exception that no esterolytic activity was demonstrable at all till the animals were fourteen days old with this substrate. The rise of esterase levels in the testes of both groups with the onset of maturity is paralleled by a similar rise in esterolytic function in other tissues involved in steroid production with the assumption of function, for example adrenal, ovary, and liver (Flint et al., 1960) and it seems that the term "non-specific esterase" is a misnomer due to our current ignorance of the true pathways of steroid metabolism.

The significance of indoxyl acetate esterase in the present results is more difficult to explain: the greatest activity of pregnenolone-3-β-ol dehydrogenase coincides with the phase of indoxyl acetate esterase activity and the two enzymes may have some relation to the synthesis route for androgen in the immature animal, but this is, at best, a hypothetical liaison.

Using various enzyme inhibitors, Aldridge (1954) has

divided carboxylic acid esterases into at least three groups, and Niemi et al. (1962) report an essentially similar distribution of α naphthyl acetate and indoxyl acetate esterases in the rat. These findings constitute an interesting contrast with the situation in the mouse where the age distribution of the two esterases is utterly different. Clearly further work is needed to clarify the relations of these enzymes one to another, and the author is convinced of their separate identities.

Since Wattenberg's (1958) description of a method for demonstrating steroid-3-β-ol dehydrogenase, the occurrence of this enzyme in the testis, ovary, and suprarenal has been confirmed by many workers. Using the substrates prenenolone and methylandrostenedial Hitzeman (1962) found that the mouse Leydig cell was strongly reactive before and immediately after birth, and noted that the intensity of the reaction had diminished considerably by the time the mice were thirty days old. The distribution of steroid-3-β-ol dehydrogenase in the present strain of mouse is similar to that described by Hitzeman in BALB/c Jax mice when pregnenolone is used as the substrate. Using 17α hydroxy pregnenolone as substrate, however, the interstitial cells are seen to have no steroid-3-β-ol dehydrogenase until the end of the tenth week of postnatal life, when more Leydig cells

react with this substrate than with pregnenolone (Table 2, Text Fig. 8). The existence of a steroid-3-β-ol dehydrogenase able to itilise 17 hydroxy progesterone has not previously been described, and the quantitative and age differences between the pictures given by the two substrates used in the present series suggest the existence of at least two separate steroid-3-β-ol dehydrogenase enzymes.

Viewed with the electron microscope, the Leydig cell presents a disappointingly stable picture between birth and adult life, in contrast with its rapidly changing histochemical characteristics. Thus, inter-digitations between adjacent cells and villous processes projecting from the cell membrane are to be seen at all ages, while the irregular lipids are constantly present; the endoplasmic reticulum and R.N.A. particles do not change with age, and there are no obvious mitochondrial alterations, either in structure or in size.

The mitochondria do not differ significantly from those described by Fawcett & Burgos (1960) in man, but they do not possess the inclusions described in the woodchuck (Christensen & Fawcett, 1961). Loops and arcs of the innermost limiting membrane of the type noted in the present material have been described by Fawcett & Burgos (1960) and their significance is not clear.

Spheroidal inclusions of the type described by these authors were not seen. It is difficult to be sure that the mitochondria with empty, structureless centres and peripheral cristae are artefact, because the membranes of these organelles are well preserved and neighbouring mitochondria may be normal in all respects. The Golgi apparatus presents no features of note.

The large irregular lipid droplets probably account for the sudanophilia of these cells when viewed with the light microscope (Baillie, 1961), but they bear no constant relation to any of the other histochemical components of the Leydig cell described in this paper. It is probable that in addition to neutral fat the cholesterol and α ketols which are present in the Leydig cells at certain are also constituents of the large irregular osmiophilic droplets. The presence of single, double, and multiple lamellae surrounding some of these droplets may represent stages in the formation of plasmalogen from simple lipid precursors. The "onion-peel" bodies or lamellated structures described by Carr & Carr (1962) and thought by them to be possibly phospholipid in nature, are almost certainly so. Comparison of the age distribution of these structures in the present series with the age occurrence of plasmalogen (Baillie, 1961) shows them to be identical, and no other histochemical component has

this peculiar age arrangement. The function of the (?) mitochondria occasionally seen in the centre of these membrane whorls is obscure.

The smaller rounded osmiophilic inclusions are apparently lipid in nature, decrease in number with age, and have no obvious histochemical counterpart. Their internal structure, if any, and significance are not understood. The groups of parallel lamellae occasionally seen in the cytoplasm do not appear to have previously been described. As other workers have noted, transition forms between mesenchymal cells and Leydig cells occur.

It has previously been shown (Baillie, 1961) that the seminal vesicles of the mouse have an S-shaped growth curve. In that study, the bulk of growth was found to take place between the fifth and seventh weeks of postnatal life, but the corresponding period of maximal growth in the present series lies between the sixth and eighth weeks of life, some seven days later than in the previous investigation. This fact, together with other subtle changes such as a steady fall in the adult body weight, causes the author to wonder whether this strain of mouse is changing slightly with time. While seminal vesicle weight is accepted by most workers as a fairly reliable indicator of androgen production, Hooker (1948)

has suggested that such target organs increase in their sensitivity to circulating androgen at puberty: more recently (Gohary et al., 1962) it has been shown that there is not a true linear relation between the seminal vesicle weight in a castrated animal and the amount of administered testosterone. Notwithstanding these reservations, it seems reasonable to conclude that androgenic steroid production rises significantly between the fifth and ninth weeks of life in the mouse.

During the same period the absolute amount and the concentration of fructose and citric acid produced by the seminal vesicle undergoes a similar rise, and Lindner & Mann (1960) have conclusively shown in the bull that this rise is closely associated with a similar rise in the testicular content of testosterone and androstenedione. The figures published by these workers show that fructose concentration in the bull between one month and adult life rises from about 4 mg. to around 400 mg. The corresponding mouse level rises from 30 mg. to over 600 mg: it is probable that if mouse vesicles were large enough to permit chemical determination of fructose before the end of the third week that the initial figures in both groups of results would be closely similar. The rise in citric acid in the mouse is not quite so marked

as that in the bull, the respective figures being 30 mg. to 200 mg. (mouse) and 4 to 300+ mg. (Bull). It is noteworthy that with advancing maturity in the mouse the ratio of the concentration of fructose to the concentration of citric acid in the seminal vesicles falls from over six to one to about three to one, i.e. it is halved. Since Lindner & Mann used so many strains of bull to compile their tables it is difficult to compare the two groups of results in this respect. While total testicular androgen rises toward maturity in the bull, the ratio of androstenedione to testosterone falls, and one is tempted to postulate a specific relation between the individual specific androgens and fructose and citric acid. The rectilinear relationship between Leydig tissue volume and seminal vesicle weight with age in the mouse (Baillie, 1961) is reminiscent of the relation between testicular androgens and seminal vesicle fructose and citric acid described by Lindner & Mann (1960).

From the quantitative histochemical data (Table 2) it is plain that the histochemical components of the Leydig cell fall into three groups:-

(1) Constituents present in fair quantities at birth and diminishing with age. Examples include the esterase capable of utilising indoxyl acetate and pregnenolone-

3-β-ol dehydrogenase.

(2) Components absent at birth, appearing in the first few weeks of postnatal life and increasing steadily with age. Cholesterol, lipofuscin, cytochrome oxidase, succinic dehydrogenase, and α naphthyl acetate esterase comprise this group.

(3) Cellular elements peculiar to the adult or nearly adult testis. Glucose, α ketols, aryl sulphatase, and 17α hydroxy pregnenolone 3-β-ol dehydrogenase.

Sudanophilia (Baillie, 1961) is common to Leydig cells of all age groups and cannot be fitted satisfactorily into this classification. Hitzeman (1962) found that lactic, malic, hydroxybuty-ic, isocitric, and succinic dehydrogenase were strongly positive at birth, weakly positive in the ensuing weeks, and strongly reactive again about puberty. These fluctuations, however, may be reflections of the relatively slow growth of the Leydig tissue in the rapidly expanding prepubertal testis (Baillie, 1961) and it seems likely that the enzymes so described by Hitzeman, together with ascorbic acid, should be considered, like sudanophilia, as forming a fourth group of histochemical constituents common to all ages. It is clear, from a consideration of the data in Table 2, that the testis of a mouse of any given age possesses its own peculiar pattern of histochemical reactions, and it should be

possible to assign an age to a given testis by comparing its histochemical attributes ("fingerprint") with Table 2. It seems likely that the histochemical pattern at birth is in part due to maternal hormonal influence, and the subsequent changes probably reflect a transition from foetal to adult steroid metabolism.

Table 3 emphasises the overall constancy of the mitochondrial and lipid distribution in the Leydig cell when seen with the electron microscope. The relationship of mitochondrial volume to the succinic acid dehydrogenase and cytochrome oxidase has already been commented on; further work is required to elucidate why the rounded lipid droplets should be reduced with age and which, if any, of the histochemical features investigated they represent.

SUMMARY.

(1) The interstitial cell of the albino mouse has been studied during the first ten weeks of life at weekly intervals using histochemical, ultrastructural, and biochemical means. Histochemical methods employed include techniques for ascorbic acid, glucose, cholesterol, lipofuscin, α ketols, cytochrome oxidase, succinate dehydrogenase, aryl sulphatase, esterases, and steroid-3-β-ol dehydrogenase. Biochemical assessment of Leydig

function depended on citric acid and fructose estimations on the seminal vesicle.

(2) The histochemical components of the Leydig cell fall into fourt categories:-

(A) Constituents present at birth and diminishing with age. Indoxyl acetate esterase and pregnenolone-3-β-ol dehydrogenase are examples. These perhaps reflect maternal humoral influence.

(B) Components absent at birth, appearing in the first few weeks of postnatal life, and increasing with age. Cholesterol, lipofuscin, cytochrome oxidase, succinic dehydrogenase, and α naphthyl acetate esterase comprise this group.

(C) Elements peculiar to adult and nearly adult Leydig cells, e.g. Glucose, α ketols, aryl sulphatase and 17α hydroxy pregneholone-3-β-ol dehydrogenase.

(D) Histochemical components common to Leydig cells of every age, e.g. sudanophilic lipids and ascorbic acid.

(3) The position of Vitamin C in the Leydig cell does not depend on either the mitochondrial location as shown by the electron microscope, or on the presence of cytoplasmic interfaces.

(4) Glucose is present in the Leydig cell; it appears at maturity.

(5) Cholesterol, initially absent from the interstitium,

appears about the second week and increases with age. It is a measure of increasing reproductive capacity.

(6) The appearance of lipofuscin in the Leydig cell appears to be related to age, lipid droplets, and P.A.S. positive granules already described. It is closely associated with Naphthol AS acetate esterase activity.

(7) α ketols are thought to be present in the adult Leydig cell.

(8) Cytochrome oxidase and succinic dehydrogenase appear in the third week of postnatal life, and their subsequent increase with age presumably reflects rising cellular energy requirements; this is not related to a rise in the number or volume of mitochondria per cell and is presumably the result of a rising enzyme content per mitochondrion.

(9) Aryl sulphatase has been demonstrated in the adult Leydig cell and its significance in steroid synthesis considered.

(10) The esterases hydrolysing α naphthyl acetate and indoxyl acetate are shown to have a different age distribution and are thought to have separate identities.

(11) A new steroid-3-β-ol dehydrogenase capable of utilising 17α-hydroxy pregnenolone is described: the age distribution of this enzyme is entirely different from

that of the enzyme capable of acting on pregnenolone.

(12) Lipid inclusions with and without surrounding membranes are described. The identity of membrane whorls with plasmalogen (phospholipid) is confirmed.

(13) Seminal vesicle weight, fructose, and citric acid are shown to have a sigmoid growth curve; the last two androgen dependent characters change at differing rates with age, and a specific relationship is postulated between testosterone and androstenedione on the one hand and fructose and citric acid on the other.

REFERENCES

ALDRIDGE, W.N. (1954). Some esterases of the rat. Biochem. J. 57, 692 - 702.

BACCHUS, H. (1950). Cytochemical study of the adrenal cortex of the rat under salt stresses. Amer. J. Physiol. 163, 326 - 331.

BAILLIE, A.H. (1961). Observations on the growth and histochemistry of the Leydig tissue in the postnatal prepubertal mouse testis. J. Anat. Lond. 95, 357 - 370.

BARNETT, S.A. & FISHER, R.B. (1943). Experiments on the silver nitrate method for histological demonstration of ascorbic acid (Vitamin C). J. Exper. Biol. 20, 14 - 15.

BOUIN, P. & ANCEL P. (1903). Recherches sur les cellules interstitielles du testicle des Mammiferes. Arch. zool. exp. gen. 1, 437 - 526.

BOURNE, G.H. (1944). Intracellular localization of Vitamin C. Nature, Lond. 153, 254 - 255.

BOURNE, G.H. (1950). Intracellular distribution of Vitamin C in the adrenal cortex. Nature, Lond. 166, 549 - 550.

CARR, I. & CARR, J. (1962). Membranous whorls in the testicular interstitial cell. Anat. Rec. 144, 143 - 148.

CHAYES, F. (1949). The theory of thin section analysis. J. Geol. 62, 92 - 101.

FAWCETT, D.W. & BURGOS, M.H. (1960). Studies on the fine structure of the mammalian testis. II. The human interstitial tissue. Amer. J. Anat. 107, 245 - 270.

FLINT, M.G., LATHE, T.R. & SILMAN, G. (1966). Changes in the microsomal enzyme systems in the liver of newborn rabbits. Biochem. J. 75, 10P.

GEDJIK, P. & BONTKE, E. (1956). Uber den Nachweis von hydrolytischen Enzymen in lipopigmenten. Z. Zellforsch. 44, 495 - 518.

GIROUD, A. (1938). "L'acide ascorbique dans la cellule et les tussus" Protoplasm, Monograph, Berlin.

GLAGOLEV, A.A. (1934). Quantitative analysis by the "Point Method". Eng. & Mining. J. 135, 399 - 400.

GOHARY, M. el., CAVAZOS, L.F. & MANNING, J.P. (1962). Effects of testosterone on histochemical reactions of epithelium of hamster ductus epididymidis and seminal vesicle. Anat. Rec. 144, 229 - 238.

GOMORI, G. (1955). Histochemistry of human esterases. J. Histochem. Cytochem. 3, 479 - 484.

GRIFFITHS, K. (1963). Personal communication.

HALLY, A.D. (1963). Personal communication.

HITZEMAN, STR. J.W. (1962). Development of enzyme activity in the Leydig cells of the mouse testis. Anat. Rec. 143, 351 - 362.

HOLT, S.J. & WITHERS, R.F.J. (1952). Cytochemical localisation of esterases using indoxyl derivatives. Nature, Lond. 170, 1012 - 1014.

HOOKER, C.W. (1948). The biology of the interstitial cells of the testis. Recent Progr. Hormone Res. 3, 173 - 196.

HUMPHREY, R.R. (1921). The interstitial cells of the iwodele testis. Amer. J. Anat. 29, 213 - 280.

LINDNER, H.R. & MANN, T. (1960). Relationship between the content of androgenic steroids in the testes and the secretory activity of the seminal vesicles in the bull. J. Endocrin. 21, 341 - 360.

LOFTS, B & MARSHALL, A.J. (1957). The interstitial and spermatogenic tissue of autumn migrants in southern England. Ibis. 99, 621 - 627.

LOISEL, G. (1904). Sur l'origine et la double significance des cellules interstitielles du testicle. Compt. rend. Soc. de. biol. 56, 448 - 452.

LUFT, J.H. (1961). Improvement in epoxy resin embedding methods. J. biophys. biochem. Cytol. 9, 409 - 414.

MANCINI, R.E., NOLAZCO, J. & BALZE, F.A. (1952). Histochemical study of normal adult human testes. Anat. Rec. 114, 127 - 148.

MOOG, F. (1943). Cytochrome oxidase in early chick embryos. J. cell comp. Physiol. 22, 223 - 231.

MULLER, G. (1955). Uber eine Vereinfachung der reaktion nach Hale. Acta Histochem. 2, 68 - 70.

NACHLAS, M.M., CRAWFORD, D.T. & SELIGMAN, A.M. (1957). The histochemical demonstration of leucine aminopeptidase. II. Histochem. cytochem. 5, 264 - 278.

NIEMI, M. & IKONEN, M. (1961). Steroid-3β-ol-dehydrogenase activity in foetal Leydig cells. Nature. 189, 592 - 593.

NIEMI, M. & IKONEN, M. (1962). Cytochemistry of oxidative enzyme systems in the Leydig cells of the rat testis and their functional significance. Endocrin. 70, 167 - 174.

OHNACKER, H.K. (1960). Der histochemische Vitamin-C Nachweis unter besouderer Berücksichtigung der Leydig-Zellen des Meerschweinchenhodens. Acta Histochem. 10, 173 - 228.

OKAMOTO, K., SHIMAMOTO, H. & SONODA, H. (1944). Quoted by Pearse,1960.

OLSON, R.E., JACOBS, F.A., RICHERT, D., THAYER, H., KOPP, L.J. & WADE, N.J. (1944). Comparative bioassay of several extracts of adrenal cortex in tests employing 4 separate physiological responses. Endocrinology. 35, 430 - 455.

PEARSE, A.G. (1960). Histochemistry, theoretical and applied. London. (Churchill).

RASMUSSEN, A.T. (1932). Interstitial cells of the testis. Cowdry's Special Cytology. 3, 1674 - 1725.

ROE, J.H. (1934). A colorimetric method for the determination of fructose in blood and urine. J. biol. Chem. 107, 15 - 22.

ROGERS, W.J. & WILLIAMS, R.H. (1947). Correlations of Biochemical and Histologic changes in the Adrenal cortex. Arch. Path. 44, 126 - 137.

ROY, A.B. (1956a). The enzymic synthesis of steroid sulphates. Biochem. J. 63, 294 - 300.

ROY, A.B. (1956b). The sulphatase of ox liver. Biochem. J. 64,651 - 657.

RUTENBERG, A.M., COHEN, R.B. & SELIGMAN, A.M. (1952). Histochemical Demonstration of Aryl Sulfatase . Science. 116, 539 - 543.

SPECK, J.F., MOULDER, J.W. & EVANS, E.A. jun. (1946). Biochemistry of malaria parasite; mechanism of pyruvate oxidation in malaria parasite. J. Biol. Chem. 164, 119 - 144.

SVIRBELY, J.L. & SZENT-GYORGI, A. (1933). The chemical nature of vitamin C. Biochem. J. 27, 279 - 285.

WATTENBERG, L.W. (1958). Microscopic histochemical demonstration of steroid-3B-ol-dehydrogenase in tissue sections. J. Histochem. cytochem. 6, 225 - 232.

ZETTERQVIST, H. (1956). The Ultrastructural Organisation of the Columnar Absorbing Cells of the Mouse Jejunum. Karolinska Institute, Stockholm.

3β-HYDROXYSTEROID DEHYDROGENASE ACTIVITY IN THE MOUSE LEYDIG CELL.

by

A.H. Baillie,
Department of Anatomy

K. Griffiths,
Department of Steroid Biochemistry.

University of Glasgow.

J. Endocrinol. In Press.

SUMMARY

One hundred and thirty-two male Swiss white mice were killed in batches of 12, between birth and the end of the tenth week of postnatal life inclusive, a total of eleven groups. Sections of the testis from every animal were incubated with three steroid substrates to demonstrate histochemically 3β-hydroxysteroid dehydrogenase. The substrates were (1) 3β, 17α-dihydroxypregn-5-en-20-one (17α-hydroxypregnenolone) (2) 3β-hydroxypregn-5-en-20-one (pregnenolone) and (3) 3β-hydroxyandrost-5-en-17-one (DHA). Using 17α-hydroxypregnenolone as a substrate no 3β-hydroxysteroid dehydrogenase activity was demonstrable in the testis until the end of the tenth week of postnatal life. With pregnenolone as a substrate 3β-hydroxysteroid dehydrogenase activity was demonstrable throughout the age series studied. It was present at birth and increased progressively until the end of the sixth week of postnatal life. Thereafter the activity decreased progressively over the ensuing four weeks. With DHA as substrate activity again was demonstrable in all age groups studied and increased progressively from birth until the end of the seventh week of postnatal life after which a relatively constant high level was maintained. On the basis of these findings the existence of more than one 3β-hydroxysteroid dehydrogenase enzyme is postulated, each enzyme being substrate specific.

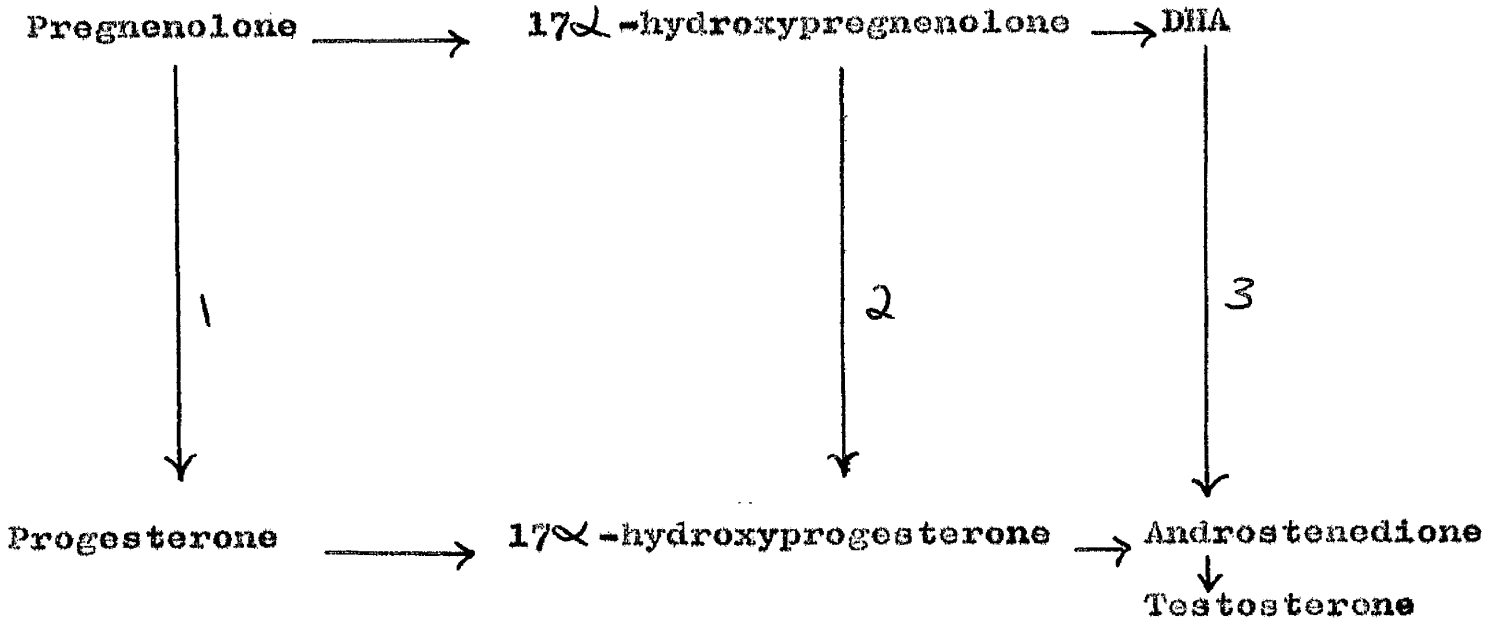
INTRODUCTION

It is considered that testosterone and androst-4-ene-3,17-dione (androstenedione) are the principle androgens produced by the mammalian testis. (Dorfman and Shipley, 1956) The production of these steroid hormones by the testis was attributed by Bouin and Ancel as early as 1903 (1903) to the Leydig cells. The actual pathways of biosynthesis of the androgens are however still not well defined. Pathways alternative to the well established one involving pregnenolone - progesterone - 17α-hydroxy-progesterone-androstenedione, have been postulated by Engel and Langer (1961) (Text fig.1).

These pathways are similar to others recently established in adrenal tissue by various groups of workers (Goldstein,Gut and Dorfmann 1960; Lipsett and Hökfelt 1961; Wolliky and Engel 1962,1963; and Mulrow,Conn and Kuljian 1962).

Reactions 1,2 and 3 (Text fig.1) all involve the conversion of a Δ^5-3 -hydroxyl group to a Δ^4-3-oxo group, and the participation of 3β-hydroxysteroid dehydrogenase (S). This enzyme, first demonstrated by Samuels, Helmreich, Lasater and Reich (1951) requires nicotinamide-adenine dinucleotide (NAD) as a co-enzyme (Beyer and Samuels 1956), and was initially demonstrated histochemically by Wattenberg (1958) by coupling the reaction via NAD-diaphorase with the reduction of a tetrazolium salt.

Text fig. 1



3β-hydroxysteroid dehydrogenase acts at sites 1-3

It would not be unreasonable to suggest the existence of substrate specific 3β-hydroxysteroid dehydrogenases which might exert some degree of control over the pathways by which the testicular androgens are synthesized. This report describes experiments designed to demonstrate histochemically, the relative activities of the "3β-hydroxysteroid dehydrogenase" of mouse testicular tissue in varying stages of development, using 17α-hydroxypregnenolone, pregnenolone and DHA as substrates.

MATERIALS AND METHODS

Altogether one hundred and thirty-two male Swiss white mice were used. They were killed in groups of twelve at weekly intervals between birth and the end of the tenth week of postnatal life inclusive.

The testes were excised within a minute of killing and placed in a beaker of crushed solid carbon dioxide. The tissues were sectioned at 15 microns in a cryostat maintained at -20°C and the sections were attached to clean dry glass slides by momentary thawing. Before incubation the slides were brought to room temperature and dried in air. Sections from each animal were incubated for half an hour in the medium described by Wattenberg (1958).

It was found that prolonged incubation had no effect on the intensity of staining, merely increasing the tendency of the sections to float. Incubations were conducted separately with the following substrates, (1) 17α-hydroxypregnenolone (2) pregnenolone and (3) DHA. The final concentration of steroid substrate in the incubation medium was 0.5 mM. Nitro BT (2, 2' - di-p-nitrophenyl - 5, 5' - diphenyl - 3, 3' - (3, 3' - dimethoxy 4, 4' diphenylene) ditetrazolium chloride) (L.Light & Co.) was employed as a final electron acceptor.

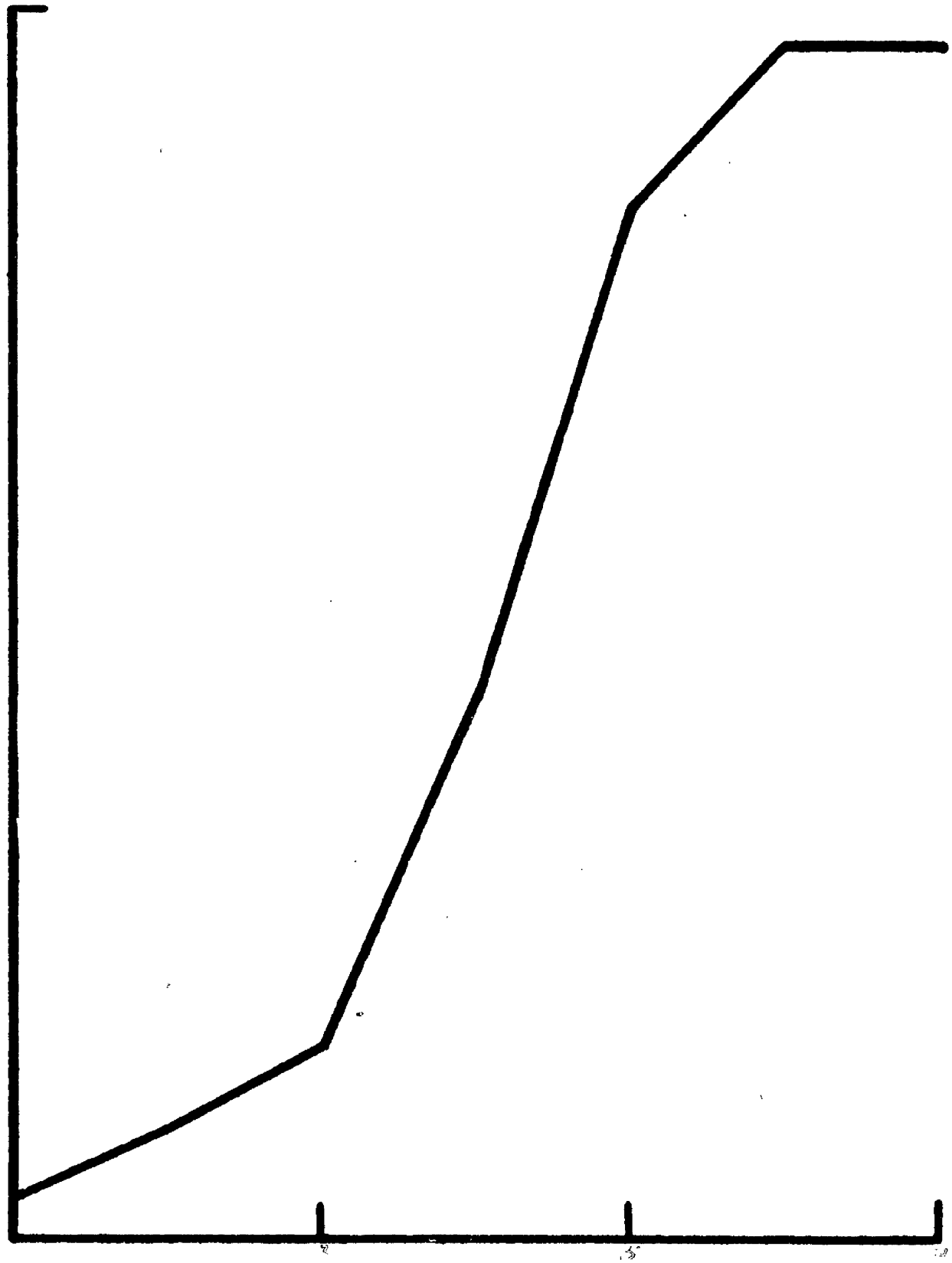
Testicular volume was measured using the formula $V = \frac{4}{3} \pi ba$ where V = testicular volume, b = half the equatorial diameter of the testis and a = half its polar diameter (Harrison and MacMillan, 1954). The measurements were carried out under a microscope using a scale calibrated in 1/100th mm.

To facilitate comparison of the three sets of results a quantitative analysis of each group of the histochemical constituents of the testis was undertaken using the point method of Glagolev (1934) and Chayes (1949). Sections at a magnification of 90 diameters were scanned with a modified grid (Hally, 1963), having points one centimetre apart on two axes at right angles to one another and the relative volume of Leydig tissue with 3β-hydroxysteroid

TABLE. I.

e in weeks.	0	1	2	3	4	5	6	7	8	9	10
sticular volume in mm^3.	0.8	6.1	18.3	31.7	88.9	169.2	195.4	196.1	196.1	196.1	196.1 (mm^3)
tertubular tissue as % of testis.	71.3	17.0	6.5	4.7	5.3	5.5	5.3	5.4	5.3	5.4	5.3 (%)
tal volume of intertubular tissue.	0.57	1.04	1.12	1.49	4.7	9.31	19.35	10.5	10.5	10.5	10.5 (mm^3)
ydig tissue capable of acting on 17 hydroxy pregnenolone as % of testis	-	-	-	-	-	-	-	-	-	-	2.4 (%)
tal volume of Leydig tissue capable of acting on 17 hydrocy pregnenolone	-	-	-	-	-	-	-	-	-	-	4.7 (mm^3)
ydig tissue capable of acting on pregnenolone as % of testis.	9.7	6.8	3.6	3.3	3.0	2.4	2.0	1.9	1.5	1.0	1.0 (%)
tal volume of Leydig tissue capable of acting on pregnenolone.	0.08	0.41	0.61	1.04	2.67	4.06	3.93	3.73	2.94	1.96	1.96 (mm^3)
ydig tissue capable of acting on DHA as % of testis.	10.3	7.3	6.5	4.4	3.0	3.0	3.0	3.0	2.9	3.0	3.0 (%)
tal volume of Leydig tissue capable of acting on DHA.	.082	0.44	1.19	1.39	2.67	5.07	5.85	5.88	5.78	5.88	5.88 (mm^3)

Text - fig. 2.



dehydrogenase was derived as a percentage. From this figure together with the figure for testicular volume the total volume of Leydig tissue having the ability to utilise a given steroid substrate was calculated.

RESULTS

(1) Testicular volume. As will be seen from text figure 2 and table 1, the testicular growth curve is sigmoid in form and growth is largely complete by the time the animal is six weeks old.

(2) 17α-hydroxypregnenolone. Histochemically the Leydig cells are completely unable to utilise this substrate until the animal reaches the end of the tenth week of postnatal life. A number of islets of Leydig tissue then become intensely reactive. (Plate 1,A). The formazan deposits take the form of minute crystals on the surface of lipid droplets contained in the Leydig cytoplasm (Plate 1,B).

(3) Pregnenolone. At birth most of the islets of Leydig tissue are intensely reactive when incubated with pregnenolone as substrate (Plate 1,C). With advancing age progressively fewer of the Leydig cells appear capable of utilising this substrate to an appreciable extent (Plate 1,D). When the adult state (Plate 2,E) is reached, only a few Leydig cells scattered in the

DESCRIPTION OF PLATE 1

3β-hydroxysteroid dehydrogenase.

A. Mouse testis aet 10 weeks after incubation with 17α-hydroxypregnenolone. X 90

B. High power view of a group of Leydig cells showing minute formazan crystals on the surface of lipid droplets in the Leydig cytoplasm. X 270

C. Neonatal testis showing intense reactivity of Leydig tissue islets with pregnenolone. X 90

D. Four week old testis after incubation with pregnenolone showing relative reduction in enzyme activity. X 90

DESCRIPTION OF PLATE 2

3β-hydrosteroid dehydrogenase.

E. Ten week testis incubated with pregnenolone showing only slight dehydrogenase activity. X 90

F. One week old testis showing intense Leydig reactivity with DHA. X 90

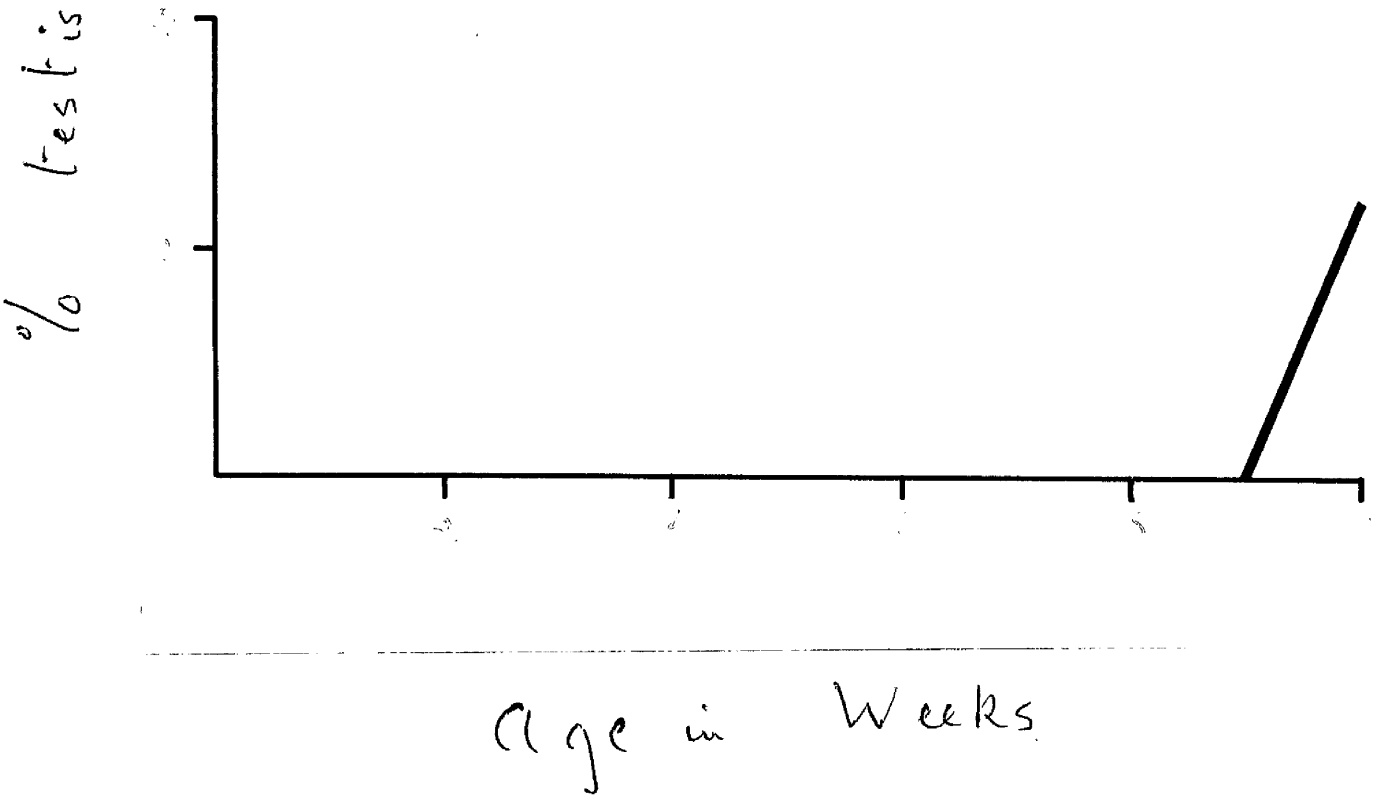
G. Five week old testis showing relative reduction of enzyme activity after DHA incubation. X 90

H. Adult (ten week) testis after DHA incubation showing two islets of intensely reactive Leydig tissue just deep to the tunica albuginea. X 90

Plate 1

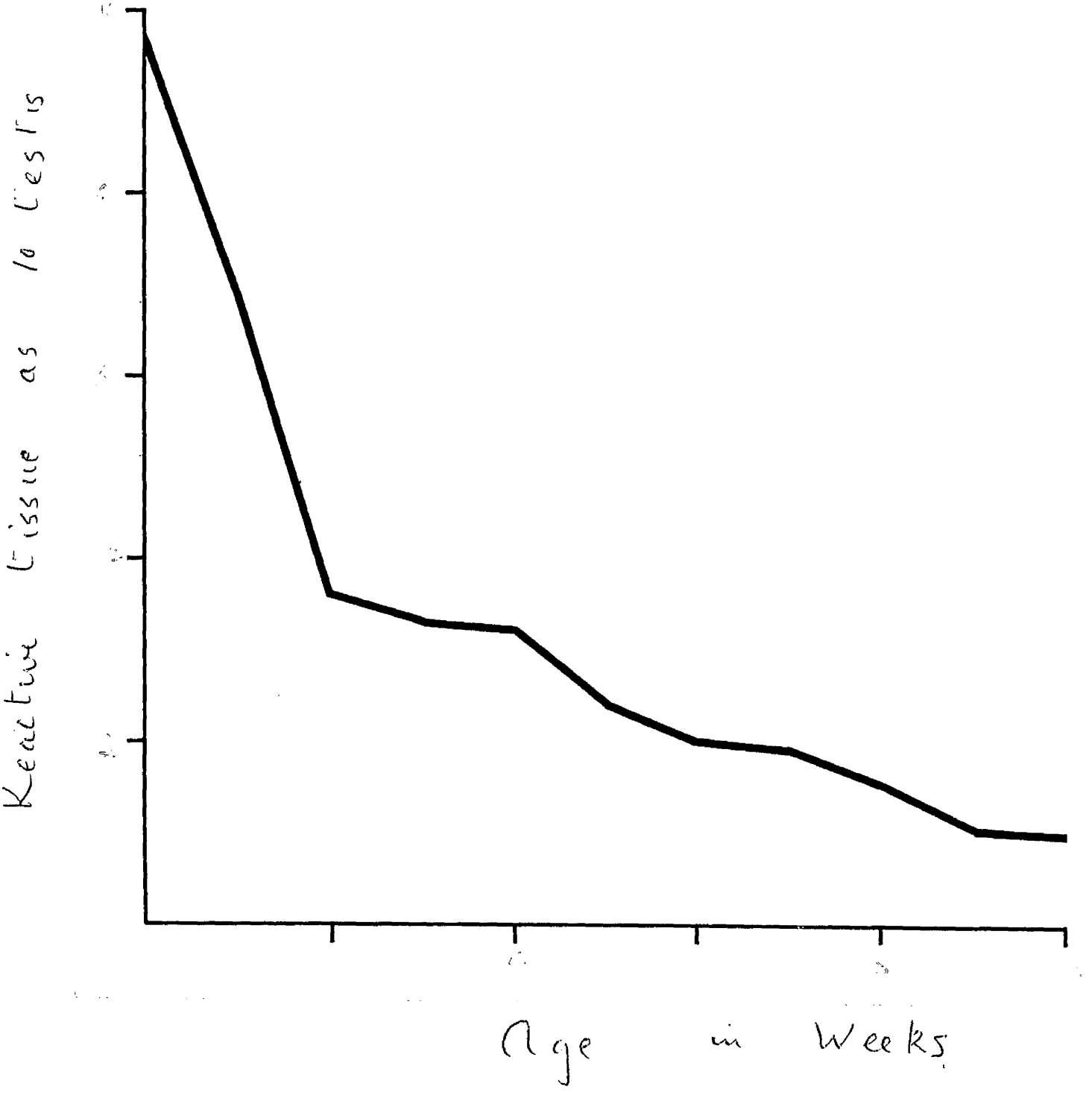
Text-fig. 3.

A. 17 α - hydroxy pregnenolone.



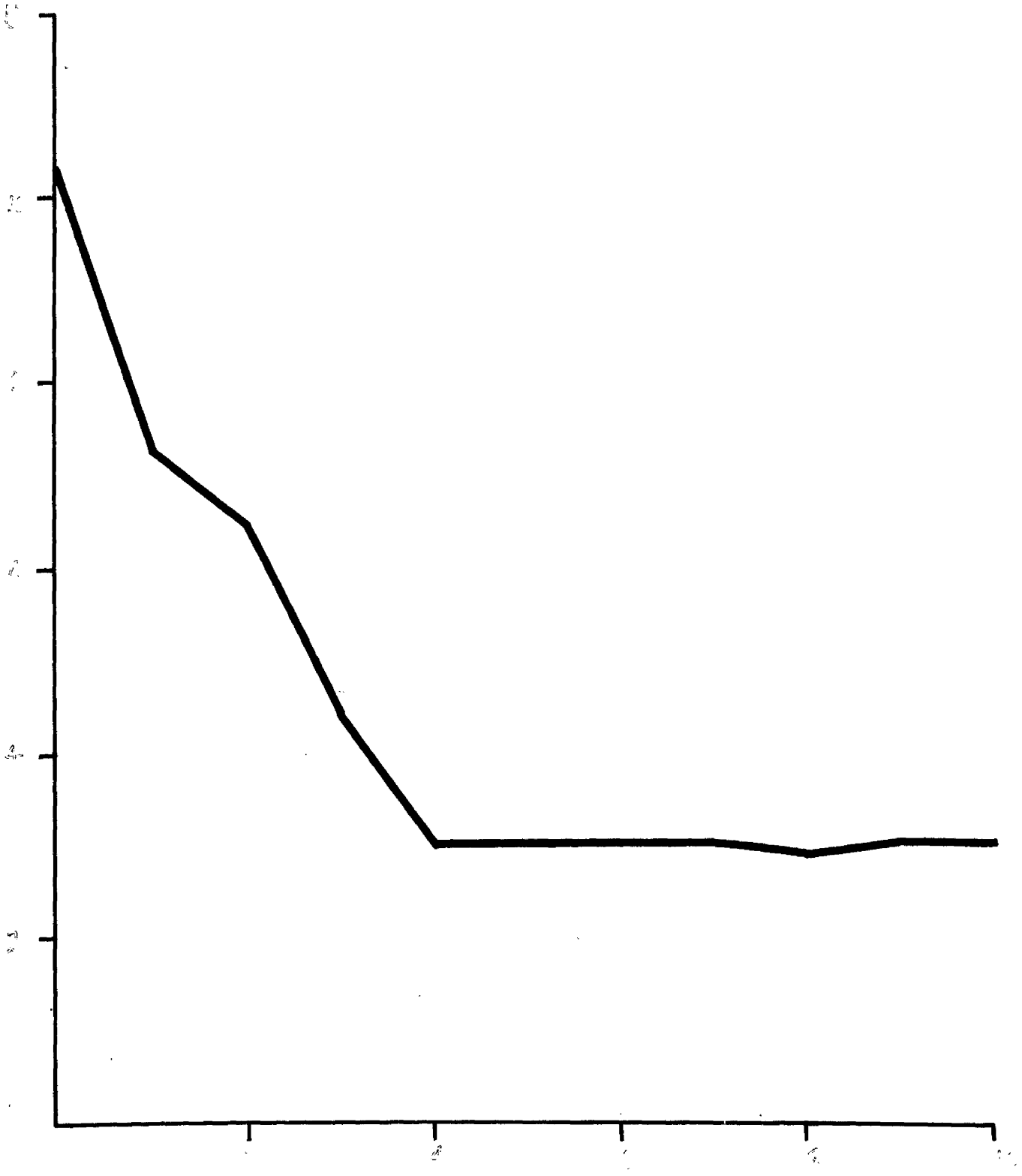
Text-fig. 3.

B. Pregnenolone.



Text - fig. 5.

C. D H A.



Age in weeks

interstitium are reactive.

(4) DHA. At birth practically the entire interstitium reacts positively when incubated with DHA. After seven days the intense reaction is maintained (Plate 2,F) and over the ensuing weeks it falls off slightly (Plate 2,G).

In the adult while some parts of the interstitium are weakly reactive a number of intensely reactive islets persist (Plate 2,H). A peculiar feature of the distribution of these intensely reactive patches lies in the fact that most of them appear to be subscapular, that is to say immediately below the tunica albuginea.

(5) Quantitative results. The quantitative data are summarised in table 1. Fluctuations in the relative volumes of reactive Leydig tissue are shown in text figure 3. From the testicular volume at the appropriate age (table 1) the relative volumes of reactive Leydig tissue can be translated into absolute volumes (Text figure 4, table 1). From Text figure 3 we see that the relative volume of reactive Leydig tissue when exposed to either pregnenolone or DHA undergoes a marked reduction in volume during the first few weeks of extra uterine life. It reaches a low level in both instances, which persists into adult life. With 17α-hydroxypregnenolone on the other hand no reactivity is demonstrable until the last

week of postnatal life studied. Reference to text figure 4 indicates that a very different state of affairs exists when the absolute volumes are considered. As in text figure 3 Leydig tissue reacting with 17α-hydroxypregnenolone is limited to the last week of postnatal life studied. Using pregnenolone the absolute volume of Leydig tissue (text fig.4, table 1) which reacts positively increases progressively with age until the fifth or sixth week of postnatal life and then falls steadily to reach a low adult level about the ninth week of postnatal life. With DHA however the absolute volume of reactive Leydig tissue has a sigmoid growth curve, the adult figure being reached by the end of the sixth week of postnatal life.

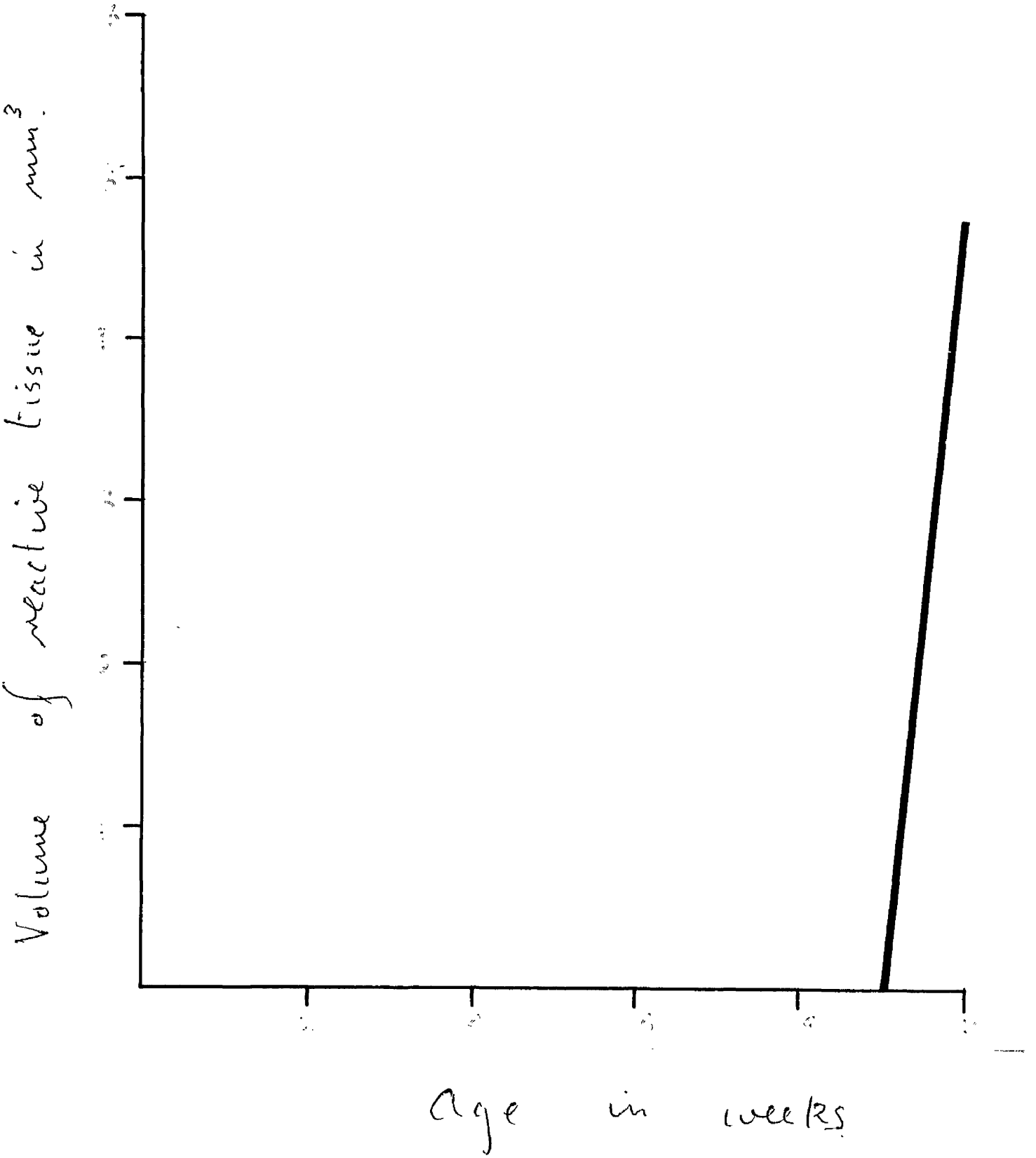
DISCUSSION

The typical sigmoid testicular growth curve is usual for this type of organ and requires no comment. 3β-hydroxysteroid dehydrogenase activity with 17α-hydroxy-pregnenolone as a substrate has not been described previously.

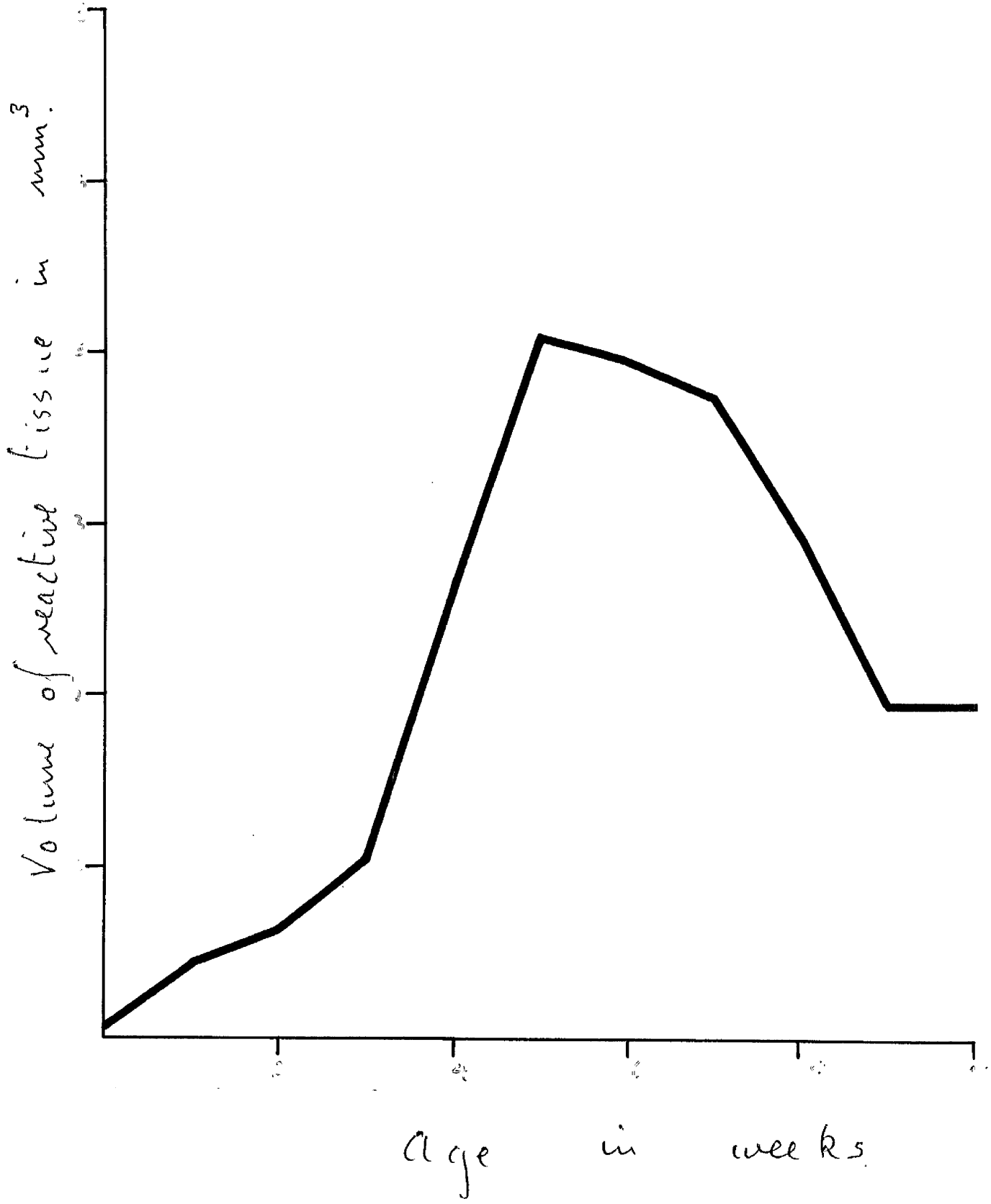
The results from incubation with DHA and pregnenolone resemble closely the original findings of Wattenberg (1958), who found that 3β-hydroxysteroid dehydrogenase was more reactive withthe former. It is clear that more Leydig

Text - fig. 4.

A. 17 α - hydroxy pregnenolone.

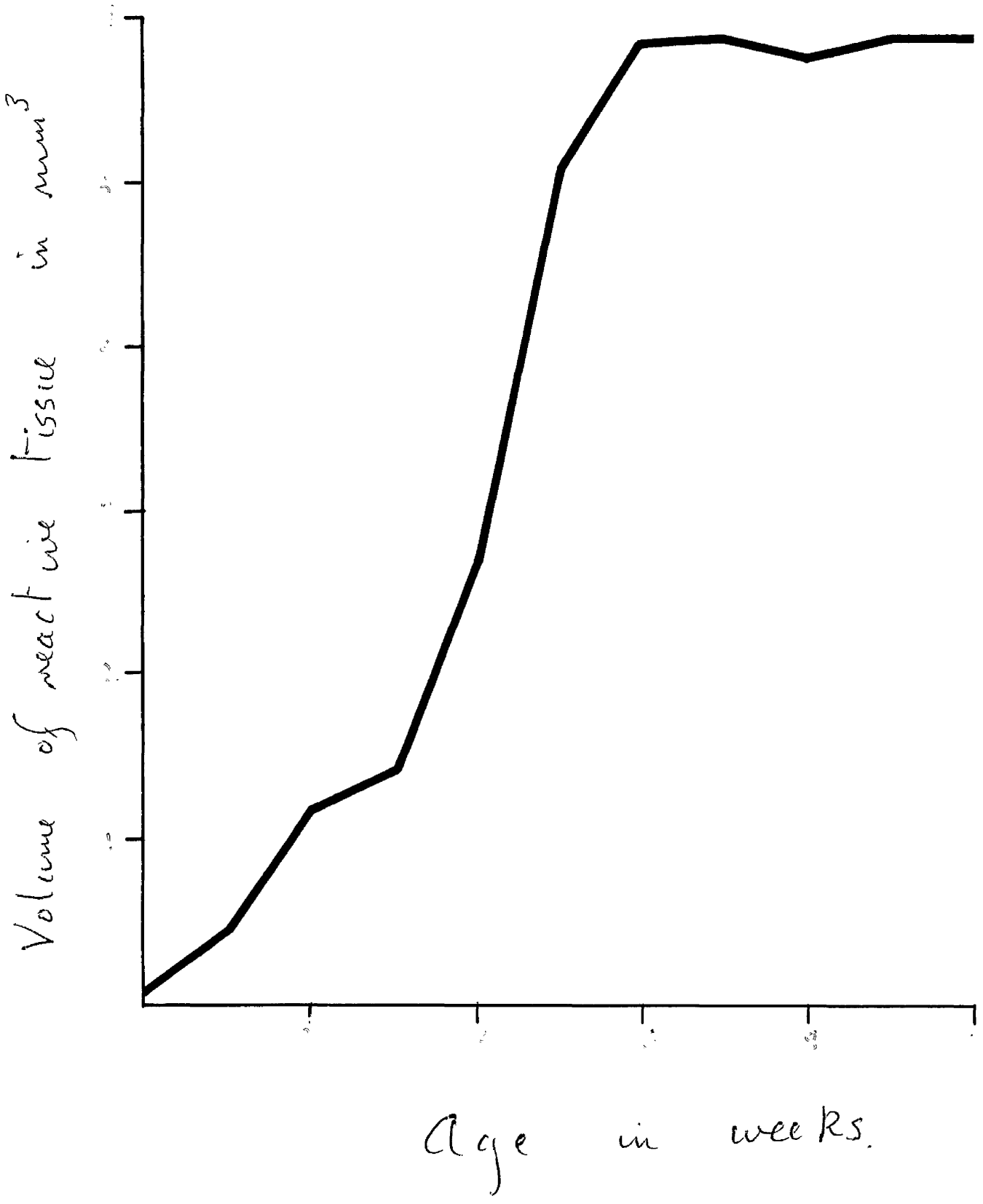


Text - fig. 4.
B. Pregnenolone.



Text - fig. 4.

C. D H A



cells have a 3β-hydroxysteroid dehydrogenase capable of using DHA than pregnenolone throughout the whole age series studied. It is interesting to note in this context that Hitzeman (1962) found that 3β-hydroxysteroid dehydrogenase was more active when presented with pregnenolone as a substrate than with a synthetic compound containing a Δ^5-3β-hydroxyl group, namely 17α-methylandrost-5-one-3β-17β-diol. Comparison of the figures giving the total volume of Leydig tissue capable of acting on pregnenolone with the total volume of Leydig tissue capable of acting on DHA indicates that at every age more Leydig cells can use DHA as a substrate for 3β-hydroxysteroid dehydrogenase than pregnenolone and this disparity in enzyme activities increases with age. These facts suggest the existence of different 3β-hydroxysteroid dehydrogenases each specific for different Δ^5-3β-hydroxysteroids. This is further supported by the fact that mouse Leydig tissue fails entirely to utilise 17α-hydroxypregnenolone until the tenth week of postnatal life. The recent report by Weliky and Engel (1963) suggests the presence of different 3β-hydroxysteroid dehydrogenases in adrenal tissue.

The graphs giving the relative volume of Leydig tissue capable of using DHA and pregnenolone (Text figure 3) compare very closely with those recently published by Niemi and Ikonen (1963). On the other hand from Text figure 4 it will be seen that the absolute volume of Leydig tissue

capable of utilising the substrate DHA increases in a regular manner during extra-uterine life. This increase is largely complete by the end of the sixth week of postnatal life and closely resembles the increase in the absolute volume of sudanophilic Leydig tissue described by Baillie (1961). There is a similar close resemblance between the absolute volume of Leydig tissue capble of using pregnenolone as a substrate for 3β-hydroxysteroid dehydrogenase and the absolute volume of Leydig tissue stainable with the plasmal reaction. It thus would appear in the growing mouse that the ability to use pregnenolone as a substrate for 3β-hydroxysteroid dehydrogenase is associated with the existence in the cells of lipids stainable with the Schiff reaction. We have no reason at present to suppose that these observations are related. In a like manner sudanophilic cytoplasmic lipids appear to be associated with the existence of a 3β-hydroxysteroid dehydrogenase capable of utilising DHA.

Although it would appear that the pathway DHA - androstenedione - testosterone is important throughout the development of the mouse testis, interpretation of these histochemical observations with regard to the pathway of androgen biosynthesis is not easy. In vitro experiments using mouse testicular homogenates are now in progress in the hope that these may provide further information on this subject.

ACKNOWLEDGMENT

The authors are grateful to Professor G.M. Wyburn and Dr. J.K. Grant for their interest and encouragement.

REFERENCES

Baillie, A.H. (1961). Observations on the growth and histochemistry of the Leydig tissue in the postnatal prepubertal mouse testis. J. Anat. 95, 357.

Beyer, K.F. & Samuels, L.T. (1956). Distribution of steroid - 3β-ol-dehydrogenase in cellular structures of the adrenal gland. J. Biol.Chem. 219, 69.

Bouin, P. & Ancel,P. (1903). Recherches sur les cellules interstitielles du testicle des Mammiferes. Arch. zool. exp. gen. 1, 437.

Chayes, F. (1949). The theory of thin section analysis. J. Geol. 62, 92.

Dorfman, R.I. & Shipley, R.A. (1956). Androgens. Wiley, New York.

Engel, L.L. & Langer, L.J. (1961). Biochemistry of steroid hormones. Ann. Rev. Biochem. 30, 499.

Glagolev, A.A. (1934). Quantitative analysis by the "Point Method". Eng. & Mining J. 135, 399.

Goldstein, M., Gut, M. & Dorfman, R. (1960). Conversion of pregnenolone to dehydroepiandrosterone. Biochem. Biophys. Acta. 38, 190.

Hally, A.D. (1963). A counting method for sectional analysis in biology. Proc. Anat. Soc. 1963.

Harrison, R.G. & MacMillan, E.W. (1954). The effects of high epididymal obstruction upon the Leydig cell volume of the rat testis. J. Endocrin. 11, 89.

Hitzman, J.W. (1962). Development of enzyme activity in the Leydig cells of the mouse testis. Anat. Rec. 143, 351.

Lipsett, M.B. & Hökfelt, B. (1961). Conversion of 17α-hydroxypregnenolone to cortisol. Experientia. 17, 449.

Mulrow, P.E., Cohn, G.L. & Kuljian, A. (1962). Conversion of 17 hydroxypregnenolone to cortisol by normal and hyperplastic human adrenal slices. J.Clin.Invest. 41, 1584.

Niemi, M. & Ikonen,M. (1963). Histochemistry of the Leydig cells in the postnatal prepubertal testis of the rat. Endocrinology, 72, 443.

Samuels,L., Helmreich,M., Lasater,M. & Reich,H. (1951). An enzyme in endocrine tissues which oxidises Δ^5-3β-hydroxysteroids to a,β - unsaturated ketones. Science, 113, 490.

Wattenberg, L.W. (1958). Microscopic histochemical demonstration of steroid-3β-ol-dehydrogenase in tissue sections. J. Histochem. Cytochem. 6, 225.

Weliky, I. & Engel,L.L. (1963). Metabolism of progesterone-4-C^{14} and pregnenolone-7α-H^3 by human adrenal tissue. J. Biol. Chem. 238, 1302.

Weliky, I. & Engel,L.L. (1962). The conversion in vitro of 17 hydroxypregnenolone to cortisol by a human adrenal tumor. J. Biol. Chem. 237, 2089.

ESTERASE SUBSTRATE SPECIFICITY IN THE TESTIS OF THE GROWING MOUSE.

by

A.H. BAILLIE.

(From the Department of Anatomy, The University, Glasgow).

Quart. J. micr. Sci. In Press.

SUMMARY

The age distribution of esterases in the interstitial cell of the mouse testis was studied between birth and the end of the tenth week of postnatal life using the substrates 3-acetyl-5-bromoindoxyl, α-naphthyl acetate, Naphthol AS acetate, Naphthol AS-LC acetate, and Naphthol AS-D acetate.

Using indoxyl acetate, esterase activity is prominent at birth and wanes over the first two weeks of postnatal life. Esterase activity using the substrates α-naphthyl acetate, Naphthol AS acetate, and Naphthol AS-LC acetate is first demonstrable two, three, and seven weeks after birth respectively, and in each instance increases steadily with age. Differences in the volume of reactive interstitial tissue for each of these substrates are demonstrable at all ages. Naphthol AS-D acetate is only utilised by the interstitial cells of the ten week old mouse.

No esterase substrate is used by all age groups, and quantitative differences at a given age exist between the individual substrates. These results are thought to imply the existence of at least five substrate specific esterases in the mouse interstitial cell, and their possible relation to steroid synthesis is noted.

INTRODUCTION

The interstitial cells of the testes of many adult mammals are known (Nachlas & Seligman, 1949; Gomori, 1946) to have esterase activity belonging to one or another of Aldridge's (1954) three types, and Huggins & Moulton (1948) have postulated a connection between "non-specific" esterase activity in the testis and male sex steroid production. It has recently been shown (Baillie & Griffiths, In press) that another enzyme involved in androgen synthesis, namely 3β-hydroxysteroid dehydrogenase, consists in not one, but at least three different enzymes, each of which is substrate specific and possesses a characteristic age distribution in the growing mouse.

Preliminary studies on interstitial cell esterase in the testis of the growing mouse, using the substrates α-naphthyl acetate and indoxyl acetate, suggested a degree of substrate specificity, although this is denied by some workers, and it was felt that the problem might repay further study.

MATERIAL AND METHODS.

Mice of the Swiss white strain, ranging in age from 0 days to 10 weeks were used in this study. In all, 220 animals were used in preparation of the age series; they

were killed in groups of 20 at birth and at weekly intervals thereafter up to and including the end of the tenth week of postnatal life. Within a minute of killing both testes were removed and placed in a beaker of crushed solid carbon dioxide. The gonads were then sectioned at 15 microns in a cryostat maintained at -20°C and the sections attached to clean dry glass slides by transient thawing. Before incubation the slides were brought to room temperature and dried in air. Sections from each animal were incubated separately with each of the following five substrates to demonstrate esterase activity:

(1) Indoxyl acetate, using the method first described by Holt & Withers (1952).

(2) α-naphthyl acetate and simultaneous coupling with Fast Blue B salt (I.C.I. Ltd.) as described by Pearse (1960).

(3) Naphthol AS acetate (G.T. Gurr) using simultaneous coupling with Fast Blue RR (G.T. Gurr) at a pH of 7.4, maintained by phosphate buffer. 4 benzoylamino-2:5-dimethoxy-aniline was the only stable diazotate which gave satisfactory results with the present material; persistently negative results were experienced with the diazotates O-aminoazotoluene, 4-chloro-O-anisidine, and 5-chloro-O-toluidine. Fast blue RR was found to give a more even

picture when dissolved in acetone rather than buffer initially.

(4) Naphthol AS-LC acetate (E. Gurr) using simultaneous coupling with Fast Garnet GBC (E. Gurr). While Fast Garnet GBC (or 4-amino 3:1-dimethylazobenzene) gave consistent results, Corinth LD (4-amino 5-chloro-O-benzanisidine) was not found to give satisfactory results with testis.

(5) Naphthol AS-D acetate (G.T. Gurr) buffered at pH 7.0 and coupling simultaneously with Fast blue BB salt (G.T. Gurr). While Fast blue BB salt (4 benzoyl-amino-2:5-diethoxyaniline) gave satisfactory results, at least with the oldest animals studies, Fast black K salt (4-amino-2:5-dimethoxy 4-nitro azobenzene) gave no positive results.

Testicular volume was measured using the formula $V = {}^{4}/_{3}\pi\ b^{2}a$ where V = the volume of the testis, b = half its equatorial diameter, and a = half its polar diameter (Harrison & MacMillan, 1954). The measurements were conducted under a microscope on unfixed testes using a scale calibrated in 1/100 mm.

To enable the five substrates to be compared, a quantitative analysis of the separate substrate results at each age was undertaken using the point method of

Glagolev (1934) and Chayes (1949). Sections at a magnification of 90 diameters were scanned with a modified grid (Hally, 1963), having points one centimetre apart on two axes at right angles to one another, and the relative volume of intertubular tissue capable of utilising a given esterase substrate was derived as a percentage. From this figure, together with the figure for testicular volume, the total volume (absolute volume) of interstitial tissue with the ability to utilise a given esterase substrate was calculated.

RESULTS

(1) Testicular volume. As will be seen from Table I, the testis grows particularly rapidly between the second and sixth weeks of postnatal life. The esterases described below are found only in the mouse interstitium; the seminiferous tubules do not have much esterase activity.

(2) Indoxyl acetate. This substrate is only utilised by the interstitial cells of the neonatal, one and two week old testis. An esterase able to deal with this substrate is present in most of the interstitial cells at birth (Fig. 1,A) and dwindles steadily over the ensuing two weeks (Fig. 1,B). The interstitial tissue of testes from animals three weeks old and over is incapable of using indoxyl acetate. The steel blue indigo precipitate is not dispersed in the

FIGURE 1. (Plate).

(A) Neonatal testis after incubation with indoxyl acetate. Most of the interstitial cells possess esterase activity.

(B) Testis, two weeks old, incubated with indoxyl acetate. Only some of the interstitial cells have esterase activity.

(C) Testis, four weeks old, after incubation with α-naphthyl acetate. Most of the interstitium has an esterase capable of acting on this substrate.

(D) Testis, six weeks old incubated with α-naphthyl acetate. The interstitial cells all have intense esterase activity.

interstitial cell lipids, but its precise cytoplasmic location is unsure.

(3) α-naphthyl acetate. The age distribution of the esterase acting on this substrate is quite different from that acting on indoxyl acetate. It is entirely absent from the neonatal testis and first makes its appearance at the end of the second week of postnatal life when a few of the interstitial cells become quite strongly positive. The majority, however, at this age are either weakly positive or negative. Over the ensuing two weeks (Fig. 1,C) practically the whole interstitium appears to develop an esterase capable of metabolising α-naphthyl acetate. Close scrutiny of the preparations at this age and later reveals considerable diffusion of the reaction products from the interstitial cells into the surrounding structures including the connective tissue ground substance, basement membranes, blood vessels, and even the periphery of adjacent seminiferous tubules. Brief incubation (5 minutes or so) reduces spread from the interstitial cells to surrounding structures, but even so it is impossible to hazard any guess as to the precise location of the esterase in the interstitial cell cytoplasm. The testes from animals aged five to ten weeks (Fig. 1,D) present a uniform picture of intense interstitial esterase activity.

FIGURE 2. (Plate).

(A) Testis, three weeks old, incubated with Naphthol AS acetate. A few interstitial cells have esterase activity.

(B) Testis, eight weeks old, Naphthol AS acetate incubation. Most of the interstitial cells are esterase reactive.

(C) Testis, nine weeks old incubated with Naphthol AS-LC acetate. Fine granules and crystals of formazan are seen in the cytoplasm of reactive interstitial cells.

(D) Testis, ten weeks old, after incubation with Naphthol AS-D acetate. Only a few interstitial cells have esterase activity.


A


C

(4) Naphthol AS acetate. Using this substrate esterase activity is not present until the animal is three weeks old, when a few of the interstitial cells exhibit some activity (Fig. 2,A). The ability to utilise this substrate increases thereafter steadily with age until the end of the eighth week (Fig. 2,B) of postnatal life, when most of the interstitial cells react positively. The reaction product is deposited in fine particles in the cell cytoplasm for the most part, but some is in solution in the cellular lipids.

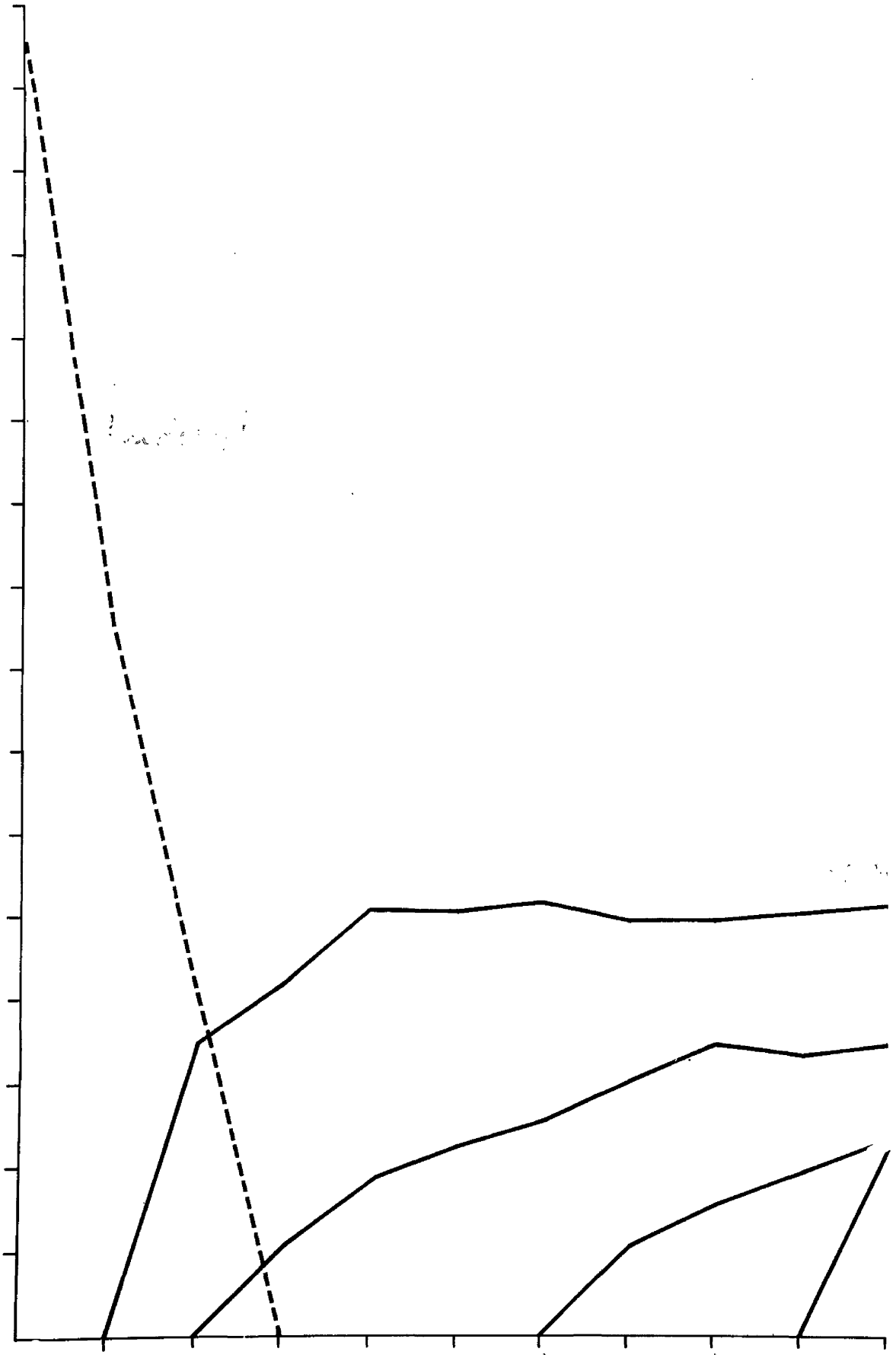
(5) Naphthol AS-LC acetate. The age distribution of esterase capable of acting on this substituted naphthol is essentially similar to that of the esterase acting on Naphthol AS acetate itself, only this esterase appears some four weeks later at the end of the seventh week of postnatal life. It never is present in such large quantities as the preceding esterase. The reactive interstitial cells contain fine granules and crystals of formazan (Fig. 2,C).

(6) Naphthol AS-D acetate. This substituted naphthol is only utilised by some of the interstitial cells of the ten week old animal (Fig. 2,D). The formazan is deposited in the form of minute crystals which do not appear to be lipid soluble.

(7) Quantitative observations. The quantitative histo-

FIGURE 3.

This graph shows the esterase active interstitial tissue for each substrate studied as a percentage of the testis during the first ten weeks of postnatal life.

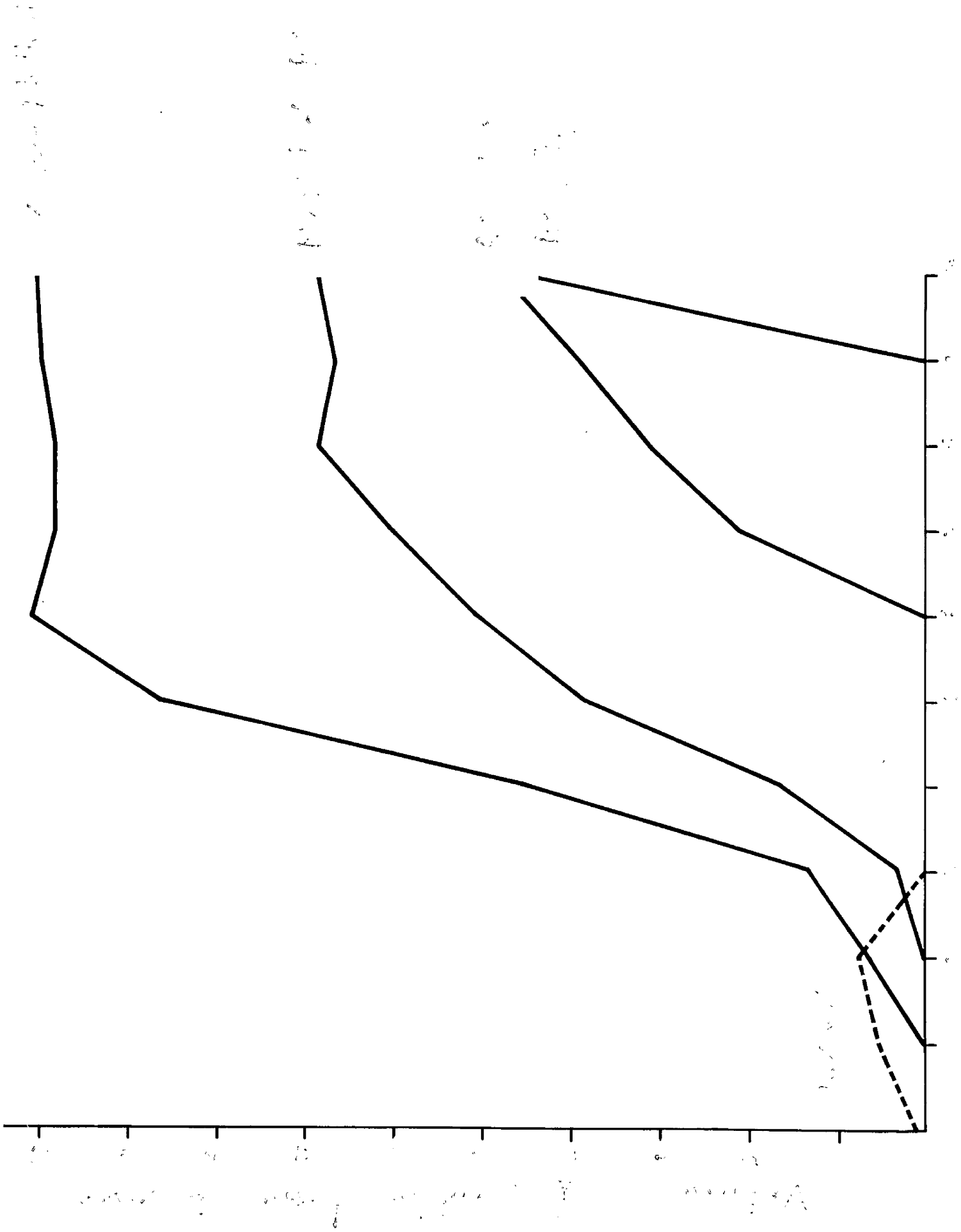


chemical data are summarised in Table I and Figures 3 and 4. From Table I and Figure 3 it will be seen that at birth 15.6% of the testis contains an enzyme capable of dealing with indoxyl acetate; over the ensuing three weeks the relative volume of interstitial tissue capable of using indoxyl acetate falls to zero. Figure 4 on the other hand translates the relative volume, i.e. the percentage of the testis comprising reactive tissue, to an absolute volume by combining the percentage with the known volume of the testis at a given age. From Figure 4 it is at once plain that the total volume of interstitial tissue capable of metabolising indoxyl acetate in fact increases slightly during the first two weeks of postnatal life. At the end of the third week of life no interstitial tissue uses this substrate.

From Table I and Figure 3 it will be seen that the relative volume of intertubular tissue capable of acting on α-naphthyl acetate increases between the end of the first and the end of the fourth weeks of postnatal life: thereafter the relative volume of reactive tissue remains fairly constant at about 5.1% of the testis. When one considers the absolute volume (Fig. 4), which allows for testicular growth, it is plain that the volume of interstitial tissue using α-naphthyl acetate grows

FIGURE 4.

This graph shows the estorase active interstitial tissue for each substrate studied as an absolute volume during the first ten weeks of postnatal life.



steadily between the first and sixth weeks of life, and this increase represented graphically gives a sigmoid growth curve.

Naphthol AS acetate esterase behaves in much the same way as α-naphthyl acetate esterase excepting that it appears a week later, completes its growth (Fig. 4) two weeks later, and is never as active as α-naphthyl acetate esterase.

The esterases acting on the substituted naphthols, Naphthol AS-LC acetate and Naphthol AS-D acetate, appear quite late, and have a peculiar and specific age distribution summarised in Figures 3 and 4. While these enzymes increase with age, the increase does not give a sigmoid growth curve.

DISCUSSION.

The typical sigmoid testicular growth curve has previously been described (Baillie, 1961) and requires no comment.

Esterase activity has been described in the interstitial cells of the testes of adult Sprague-Dawley rats (Nieni, Härkönen & Kokko, 1962) using 0-acetyl-5-bromoindoxyl acetate as substrate. Comparison of the present results with the rat findings indicates a marked species difference in that the enzyme capable of dealing

with O-acetyl-5-bromoindoxyl acetate is peculiar to the immediately postnatal mouse testes and is wholly absent from adolescent and adult testes. O-acetyl-5-bromoindoxyl acetate esterase is present during the first fourteen days only of postnatal life in the mouse testis, and it is interesting to note that this period corresponds with the phase of maximal activity of the 3β-hydroxysteroid dehydrogenase acting on pregnenolone (Baillie & Griffiths, In Press).

The presence of an esterase using α-naphthyl acetate has been recorded with mouse testis by Market & Hunter (1959), and the age distribution of α-naphthyl acetate esterase in Swiss white mice corresponds with Hitzeman's (1962) experience in DALB/c Jax mice, with the exception that in the Swiss white strain no esterolytic activity was demonstrable at all until the mice were fourteen days old. α-naphthyl acetate esterase is present in the interstitial cells of adult Sprague-Dawley rats (Niemi et al., 1962) but the position is unclear with regard to growing rats. Niemi and Ikonen (1963) have studied the distribution of a "nonspecific esterase" in the postnatal prepubertal rat using a simultaneous azo-coupling method and have recorded the presence of such an enzyme at all ages between birth and adult life, but these

writers do not state the substrate actually employed.

Of the substrates used in the present investigation, α-naphthyl acetate gives an age distribution which most nearly resembles the results in the growing rat of "non-specific esterase". Having regard to the wide variations of esterase activity established at different ages with the various substrates in the present investigation, it no longer seems adequate to speak of "nonspecific esterase" and the substrate employed should be specified.

An esterase capable of utilising Naphthol AS^{D} acetate has been described in the adult rat testis by Nieuwkoop et al., (1962) and the adult mouse and rat are similar in this particular.

Gomori (1952) stated that esterase activity in acetone fixed material showed certain site differences, depending on whether α-naphthyl acetate or Naphthol AS acetate was used, and he went on to suggest the existence of a specific Naphthol AS esterase on this basis. Reference to Figures 3 and 4 indicates that α-naphthyl acetate is used as an esterase substrate a week before the interstitial cells can utilise Naphthol AS acetate, five weeks before they can use Naphthol AS-LC acetate and eight weeks before they can utilise Naphthol AS-D acetate. These facts

suggest that not only may there be different esterases for α-naphthyl acetate and Naphthol AS acetate as postulated by Gomori, but there may even be different enzymes for each of the substituted Naphthols. This conclusion receives further support from the quantitative differences in esterase activity at any selected age when different substrates are used. The writer is satisfied that so far as the substituted Naphthols are concerned, diffusion of the final reaction product from the site of esterase activity is not occurring to any significant extent in this material. This is certainly not true of the sections incubated with α-naphthyl acetate, and the quantitative differences (Fig. 4) between these sections and those incubated with Naphthol AS acetate are due in part to diffusion artefact. Perhaps some of the discrepancies between Gomori's (1952) work on these two substrates and Pearse's (1954) findings lie in the fact that proneness to diffusion is affected by the different methods of preparation employed by the two workers. It is interesting to note that Burstone (1957) and Goessner (1958) found no essential differences in esterase activity when different substituted Naphthols were used.

In the rat (Niemi & Ikonen, 1963) about 10% of the

testis is esterase reactive at birth, and this falls to about one per cent during the second and third weeks of postnatal life. Thereafter, more of the rat testis becomes esterase reactive until the adult picture is reached. These findings closely resemble the present mouse data in so far as an esterase of one sort or another is present in 15% of the testis at birth, in three or four per cent of the testis during weeks one to three, and esterase levels thereafter rise generally to reach adult levels, indoxyl acetate esterase excepted. These esterase findings resemble closely the figures published for Sudan stained lipids expressed as a percentage of the testis in the mouse (Baillie, 1961), and one might, if considering percentages alone, decide that the interstitial cells were becoming reduced in number during the animal's growing phase. When allowance is made for the increase in the size of the testis, however, i.e. when the percentage (Fig. 3) is translated into an absolute volume (Fig. 4) then the esterase reactive interstitial tissue shows a sigmoid growth curve (with α -naphthyl and Naphthol AS acetates) and there is no suggestion of the existence of two generations of interstitial cells. Niemi and Ikonen's (1963) rat findings might perhaps be found to support this view if rat

testicular volume at the age points studied were known and absolute volumes, rather than percentages, of esterase reactive interstitial tissue calculated.

It is thought that testosterone and androst-4-ene-3, 17-dione (androstenedione) are the principal androgens produced by the mammalian testis (Dorfman & Shipley, 1956) and the proportions of androstenedione to testosterone produced are known to change with advancing maturity in the bull (Lindner & Mann, 1960). It has been shown (Baillie & Griffiths, In Press) that substrate specific 3 β-hydroxysteroid dehydrogenases vary with age in the growing mouse in much the same way as the substrate specific esterases vary with age, and it may be that the changes in these enzyme systems are related to a changing preponderance in the various pathways known to be involved in androgen synthesis.

The author is grateful for the research facilities provided in the Anatomy Department of Glasgow University.

REFERENCES

ALDRIDGE, W.N., 1954, Biochem. J. 57, 692.

BAILLIE, A.H., 1961, J. Anat. Lond., 95, 357.

BAILLIE, A.H., & GRIFFITHS, K., 1963, J. Endocrin. In Press.

BURSTONE, M.S., 1957, Arch. Path., 63, 164.

DORFMAN, R.I., & SHIPLEY, R.A., 1956, Androgens. Wiley, New York.

GLAGOLEV, A.A., 1934, Eng. & Mining J., 135, 399.

GOMORI, G., 1946, Arch. Path., 41, 121.

GOMORI, G., 1952, Microscopic Histochemistry, Univ. Press, Chicago.

GOESSNER, W., 1958, Histochemie, 1, 48.

HALLY, A.D., 1963, Personal communication.

HARRISON, R.G., & MacMILLAN, B.W., 1954, J. Endocrin., 11, 89.

HITZMAN, Str. J.W., 1962, Anat. Rec., 143, 351.

HOLT, S.J., & WITHERS, R.F.J., 1952, Nature, Lond., 170, 1012.

HUGGINS, C., & MOULTON, S.H., 1948, J. Exp. Med., 88, 169.

LINDNER, H.R., & MANN, T., 1960, Endocrinology, 21, 341.

MARKET, C.L., & HUNTER, R.L., 1959, J. Histochem., Cytochem., 7, 42.

NACHLAS, M.M., & SELIGMAN, A., 1949, Anat. Rec., 105, 677.

NIEMI, M., HÄRKÖNEN, M., & KOKKO, A., 1962, J. Histochem. Cytochem., 10, 186.

NIEMI, M., & IKONEN, M., 1963, Endocrinology, 72, 443.

PEARSE, A.G.E., 1954, Int. Rev. Cytol., 3, 329.

PEARSE, A.G.E., 1960, Histochemistry, Theoretical and Applied. Churchill, London.

AGE CHANGES IN THE MITOCHONDRIA AND SUCCINOXIDASE SYSTEM OF THE LEYDIG CELL.

by A.M. Dalllie

From the Department of Anatomy,
University of Glasgow,
Scotland.

Zeit. für Zellf. In Press.

Burstone (1959), Conklin, Dovey and Kahn (1962) and Ogawa and Shinonaga (1962) have variously shown that the sites of histochemically demonstrable cytochrome oxidase and succinic dehydrogenase activity correspond with cytoplasmic organelles otherwise indistinguishable from mitochondria. Moreover, a close correlation has been demonstrated between mitochondrial morphology, as disclosed by the electron microscope, on the one hand and succinoxidase activity, estimated by biochemical assay, on the other in the proximal convoluted tubules of the kidneys of rats in experimentally induced nephritis (Wallace, 1960). Many changes occur between birth and adult life in the lipids (Baillie, 1961), esterases (Nieuwkoop et al., 1963) and 3β-hydroxysteroid dehydrogenases (Baillie and Griffiths in Press) of the Leydig cell of the testis and these are associated with increasing androgen production (Hooker, 1948; Lindner and Mann, 1960). This report attempts to relate similar changes in the distribution of succinic dehydrogenase and cytochrome oxidase to the mitochondrial pattern revealed by the electron microscope.

Material and Methods

The animals used in this investigation were male Swiss White mice aged from birth to ten weeks. One hundred and ten animals were used in compiling the succinic dehydrogenase and cytochrome oxidase series. They were killed in groups of ten at weekly intervals between birth and the end of the tenth week of postnatal life, a total of eleven groups. Immediately after sacrifice one testis from each animal was "snap frozen" in solid carbon dioxide. The tissues were sectioned at 20 μ on a cryostat at -20°C and the sections attached to clean dry glass slides by momentary thawing. Succinic dehydrogenase activity was sought in sections from each animal using the method of Nachlas, Tsou, de Souza, Cheng and Seligman (1957); Nitro-BT was the final electron acceptor. Cytochrome oxidase was demonstrated using the method of Moog (1943) modified by Nachlas, Crawford, Goldstein and Seligman (1956).

In preparation of the ultrastructure age series eleven animals were killed singly at weekly intervals between birth and the end of the tenth week of postnatal life. Pieces of testis, about 1 mm cube in size were fixed in 1% buffered osmic acid (Zetterqvist, 1956) dehydrated in methanol, embedded in araldite (Luft, 1961) and sectioned on Porter-Blum and Huxley microtomes.

After staining with 1% methanolic lead acetate the sections were screened on uncoated copper grids using a Philips electron microscope EM 75b.

Testicular volume and Leydig tissue volume for this strain has previously been established (Baillie,1961) for each of the age points selected and the results are presented in Table 1 for the sake of clarity. In order to compare the age changes in the two enzymes with mitochondrial age changes a quantitative analysis at each age of the histochemical and ultrastructural preparations was made using the point method of Glagolev (1934) and Chayes (1949). Photomicrographs of histochemical sections at a magnification of 90 diameters were scanned with a modified grid (Hally,1963) and the Leydig tissue with succinic dehydrogenase or cytochrome oxidase was derived as a percentage of the testis. From the percentage and the testicular volume at a given age the volume of reactive tissue was calculated. In the same way many electron micrographs of Leydig cells at low magnifications were scanned and the mitochondria derived as a percentage of the Leydig tissue. The total volume of mitochondria in the Leydig tissue at a given age was derived from the percentage and the Leydig tissue volume.

RESULTS

No succinic dehydrogenase could be detected in the interstitial cells of the mouse testis before the end of the third week of postnatal life when a few Leydig cells are seen to contain small blue cytoplasmic aggregations of diformazan (Fig. 1). At this age the diformazan deposits take the form of elongated crystals for the most part and there is little evidence of formazan pooling in cytoplasmic lipids. As in all later preparations, succinic dehydrogenase activity is present in the seminiferous tubules. As the animal matures more of the interstitial cells come to have succinic dehydrogenase activity and the tendency of the reaction products to pool in cellular lipids so increases that eventually deep blue coloured lipid droplets obscure most of the cytoplasmic detail and few of the elongated crystals are to be seen (Fig. 2). Notwithstanding the progressive increase in the amount of succinic dehydrogenase activity with age in even the oldest testes examined less than half of the Leydig tissue present contains demonstrable activity.

Cytochrome oxidase activity was also absent from the interstitial tissue of the testes from the first three age groups studied. Testes from three week old mice possess abundant Leydig cell cytochrome oxidase activity

Figure 1. Testis, three weeks. A few small diformazan crystals denote succinic dehydrogenase activity in the interstitial cells.

Figure 2. Testis, eight weeks, succinic dehydrogenase. The reaction products are pooled in intracellular lipids.

Figure 3. Testis, three weeks, cytochrome oxidase. The indophenol blue is extremely widely dispersed in the Leydig lipids.

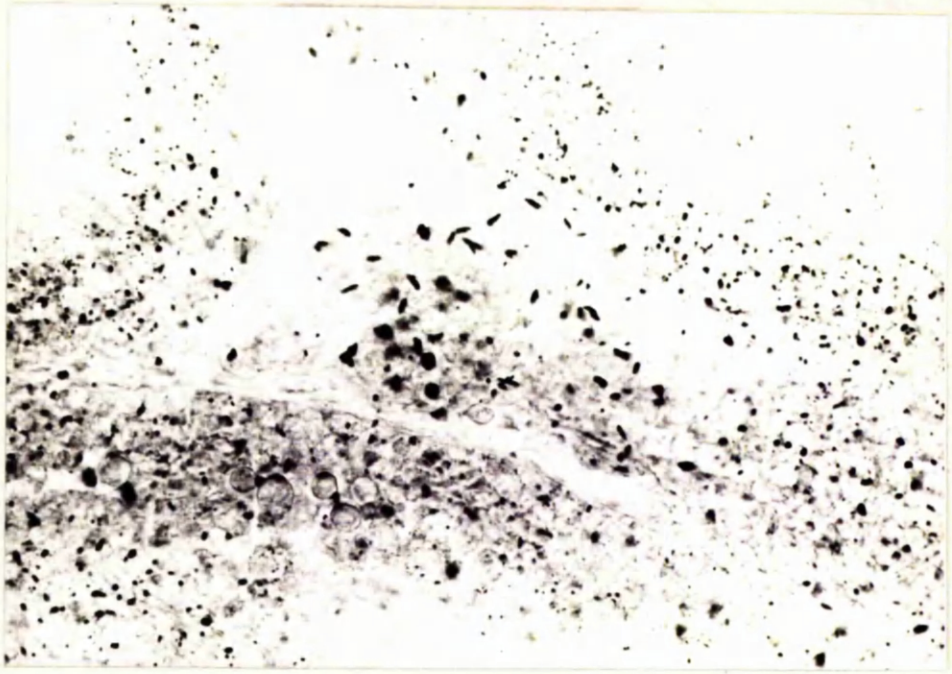


Fig 1.

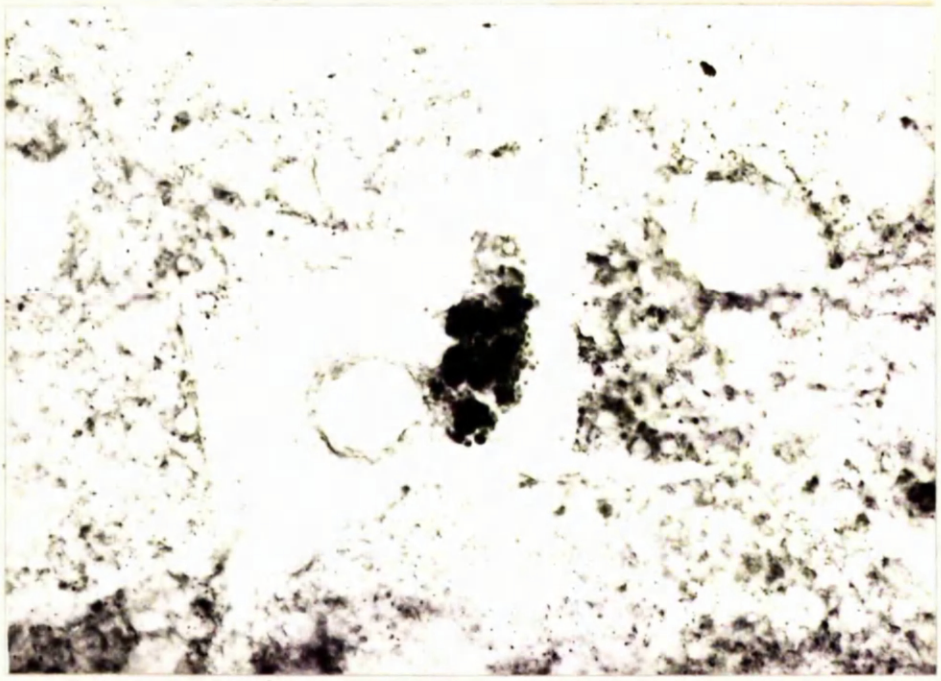


Fig 2.

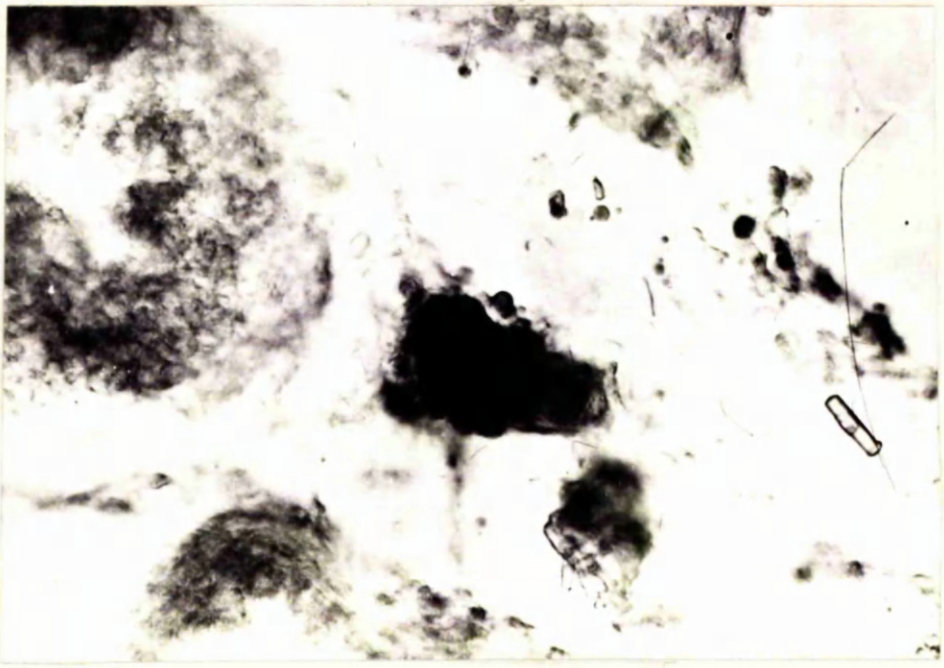


Fig 3.

and most of the interstitial tissue is coloured a deep blue. The Indophenol blue resulting from the reaction is extremely widely dispersed in the Leydig lipids (Fig. 3) and reduction of the incubation period did nothing to minimise the dispersion of the dye, merely reducing the intensity of the colour. In older testes an essentially similar picture results with most of the interstitium reactive and most of the reaction products in solution in lipids (Fig.4).

Mitochondria are present in the Leydig cells of all the mice studied and show no changes with age; a common description is thus applicable to all groups. Mitochondria constitute a fair proportion of the Leydig cells and as a rule are either oval or rounded in shape and of a more or less uniform size (Fig.5). Occasional elongated deviants occur. The internal mitochondrial structure presents few features of note. There are outer and inner limiting membranes and tubular cristae are present, formed by invaginations of the inner membrane. The interior not filled by cristae contains a matrix of moderate electron density. Bifurcation of the inner lamina is occasionally seen. Some mitochondria exhibit fixation damage and are swollen to three or four times their usual size: the interior of such damaged mitochondria contains cristae in various stages of disintegration. (Fig.6).

Figure 4. Testis, eight weeks. Most of the interstitium contains cytochrome oxidase activity.

Figure 5. Typical Leydig mitochondria are either round or oval with tubular cristae.

Figure 6. A damaged mitochondrion: these may occur while nearby organelles are well preserved.

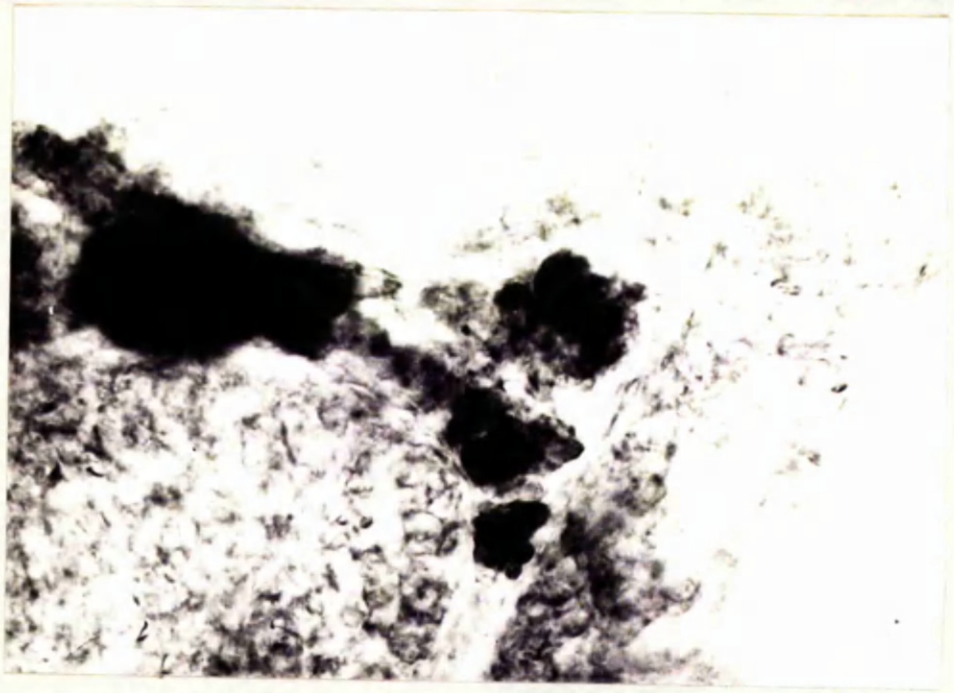


Fig. 4.

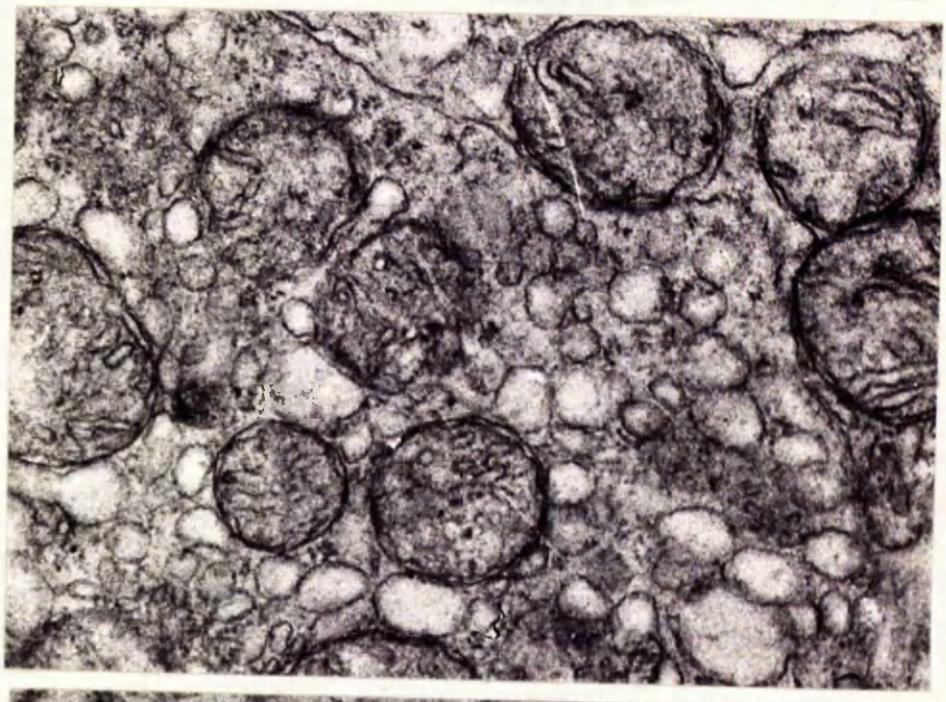


Fig. 5

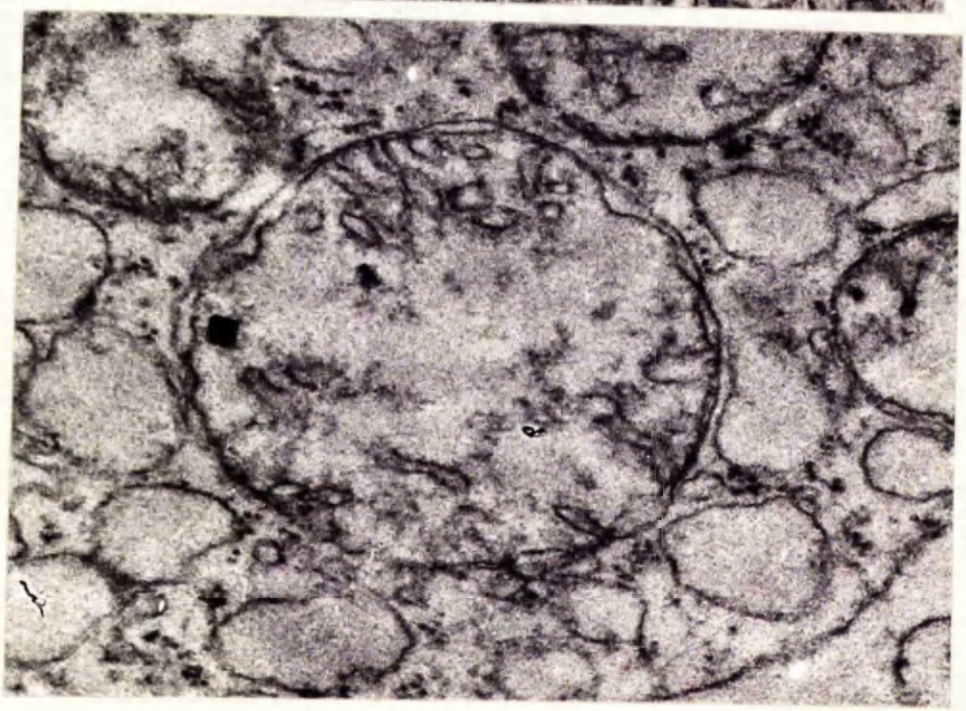


Fig. 6.

TABLE 1

Age in Weeks	0	1	2	3	4	5	6	7	8	9	10	
Average testicular volume in mm^3	0.8	6.1	18.3	31.7	88.9	169.2	195.4	196.1	196.1	196.1	196.1	mm^3
Leydig tissue volume in mm^3	0.08	0.13	0.37	0.7	3.2	7.6	8.8	8.8	8.8	8.8	8.8	mm^3
Leydig tissue containing succinic dehydrogenase as a percentage of the testis	-	-	-	0.6	0.7	1.0	1.1	1.0	1.2	1.0	1.2	%
Volume of Leydig tissue containing succinic dehydrogenase in mm^3	-	-	-	0.19	0.59	1.6	2.15	1.95	2.34	1.95	2.34	mm^3
Logarithm of volume of Leydig tissue containing succinic dehydrogenase	-	-	-	-0.7212	-0.2291	0.2041	0.3324	0.2900	0.3692	0.2900	0.3692	
Leydig tissue containing cytochrome oxidase as a percentage of the testis	-	-	-	2.2	3.0	4.2	4.4	4.3	4.3	4.4	4.6	%
Volume of Leydig tissue containing cytochrome oxidase in mm^3	-	-	-	0.7	2.67	6.2	8.6	8.41	8.41	8.6	9.0	mm^3
Logarithm of volume of Leydig tissue containing cytochrome oxidase	-	-	-	-0.1549	0.4265	0.7924	0.9345	0.9248	0.9248	0.9345	0.9542	
Mitochondria as a percentage of the Leydig cells	9.2	9.1	9.4	9.3	9.3	9.5	9.5	9.6	9.5	9.6	9.4	%
Total volume of Leydig mitochondria in mm^3	0.007	0.012	0.035	0.065	0.297	0.722	0.836	0.845	0.836	0.845	0.827	mm^3
Logarithm of total volume of Leydig mitochondria	-2.1549	-1.9208	-1.4559	-1.1871	-0.5272	-0.1415	-0.0778	-0.0731	-0.0778	-0.0731	-0.0825	

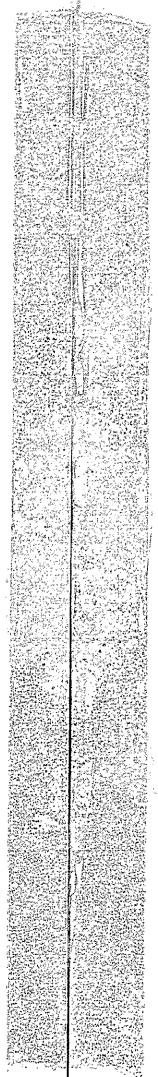
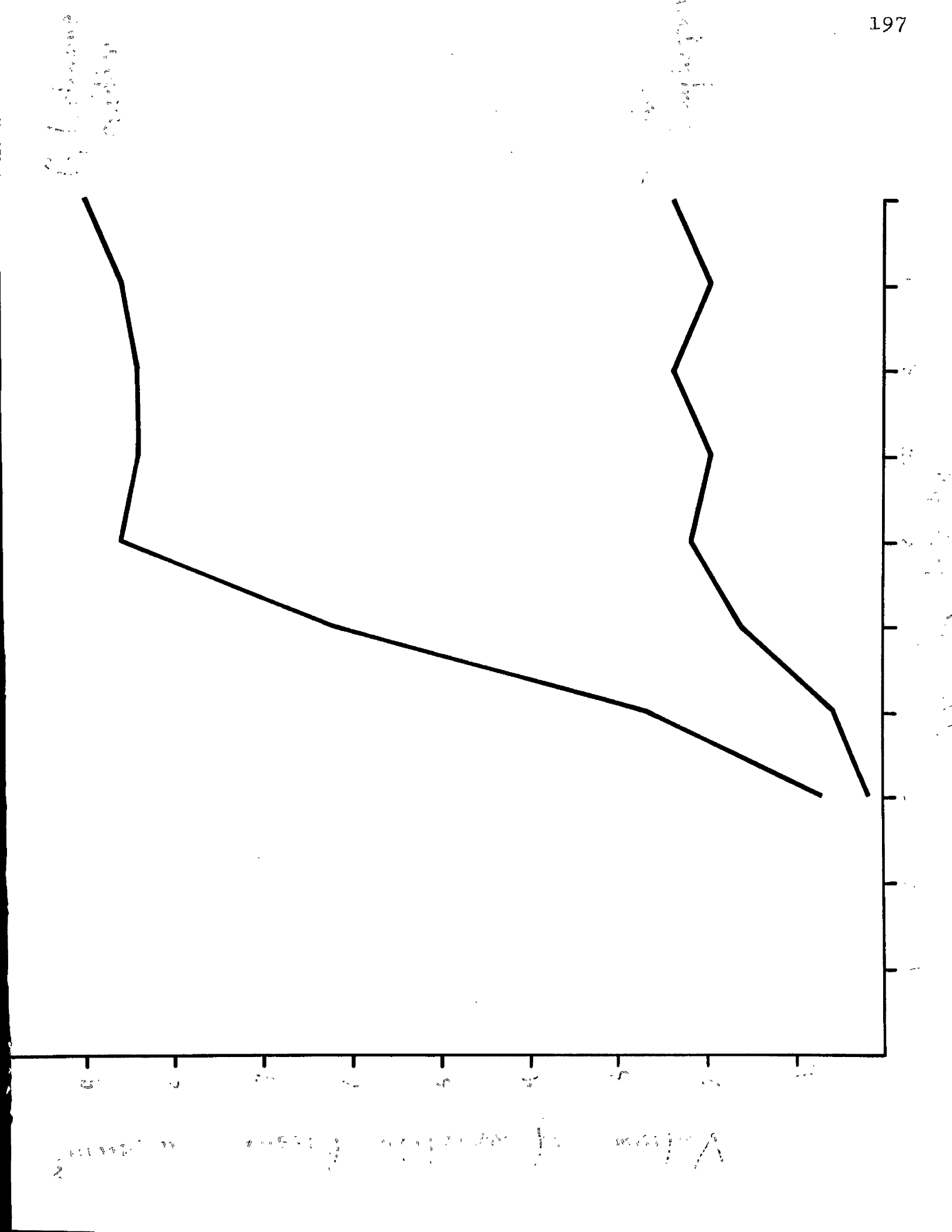
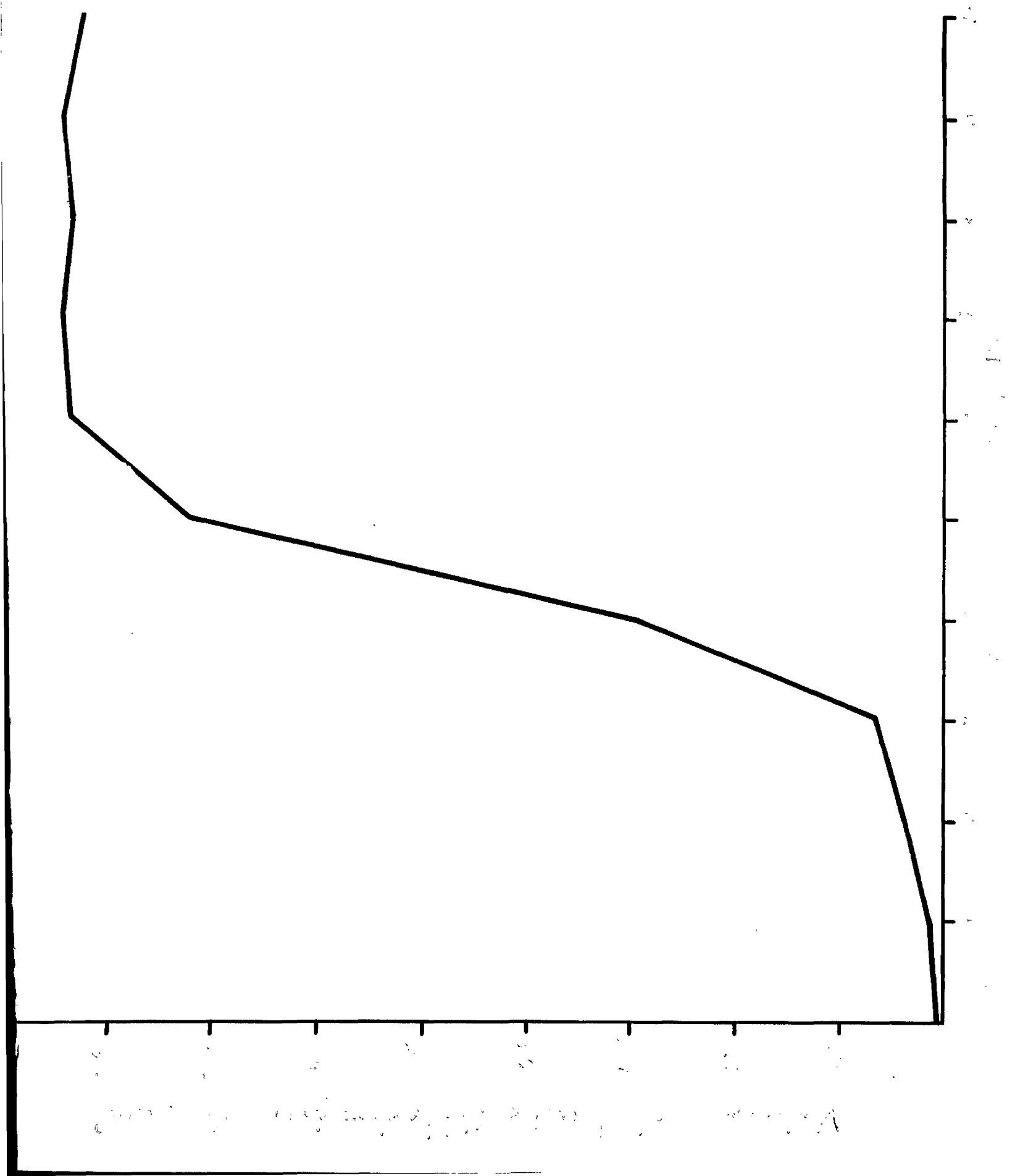


Figure 7. The graphs denote the volume of Leydig tissue with cytochrome oxidase and succinic dehydrogenase at the ages studied.



The quantitative data are summarized in Table 1. From Table 1 and Fig. 7 it is plain that the volume of Leydig tissue containing succinic dehydrogenase (not demonstrable prior to the end of the third week of postnatal life) increases steadily during the fourth, fifth and sixth weeks of postnatal life. Thereafter a plateau (Fig.7) is reached which persists into adult life. Cytochrome oxidase positive Leydig tissue behaves in a similar manner, the enzyme being first histochemically detectable at age 21 days, and the volume of reactive Leydig tissue increasing steadily between the third and the end of the sixth week of postnatal life when a stable plateau is reached. During the period studied mitochondria constantly represent about 9 per cent or so of the Leydig cell and no variation of note occurs (Table 1). Figure 8 indicates that the total volume of mitochondria in the Leydig tissue (derived from the percentage and the Leydig tissue volume) increases according to a sigmoid growth curve and that the bulk of the increase takes place between the third and the sixth week of postnatal life.

Figure 8. This graph denotes the total volume of Leydig mitochondria at the ages studied.



DISCUSSION

Nitro - BT was first successfully employed by Nachlas et al (1957) as the final electron acceptor in the cytochemical demonstration of succinic dehydrogenase and while these writers examined the testes of rats and dogs with this technique they do not record the existence of the enzyme in the interstitium. Hitzeman (1962) similarly reports great difficulty in demonstrating this specific enzyme in the Leydig cells of Balb/c Jax mice. Niemi et al (1962) on the other hand report abundant succinic dehydrogenase activity in the interstitial cells of Sprague-Dawley rats and the Leydig cells of the Swiss white mouse strain clearly resemble Sprague-Dawley rats in this respect.

The use of Nitro - BT as the coupling agent of choice in the demonstration of succinic dehydrogenase rests in the relative insolubility in lipid of this ditetrazole and the crystal deposition in the Leydig cells of the three week old mice is in accord with other authors' experience (Nachlas et al., 1957; Ogawa and Shinonaga, 1962; Chatterjee and Mitra, 1962). It is difficult to reconcile the undoubted pooling in lipids of the coloured reaction product in the older testes studied (Fig.2) with the findings of these other workers, more particularly when it is more or less universally agreed that succinic

dehydrogenase is peculiar to mitochondria (Ogawa and Shimomaga, 1962; Conklin, Dewey and Kahn, 1962). Recently, however, evidence has been adduced for an extra mitochondrial locus or loci for some succinic dehydrogenase activity, the nucleolus in particular being specified (Chatterjee and Mitra) in squamous and malignant epithelial cells; lipid droplets might perhaps come into this category but the author feels that the solubility of Nitro - BT (2, 2'-di-p-nitrophenyl-5,5' -diphenyl -3,3' [3,3'-dimethoxy -4,4' -biphenylene] ditetrazolium chloride) is not yet fully understood.

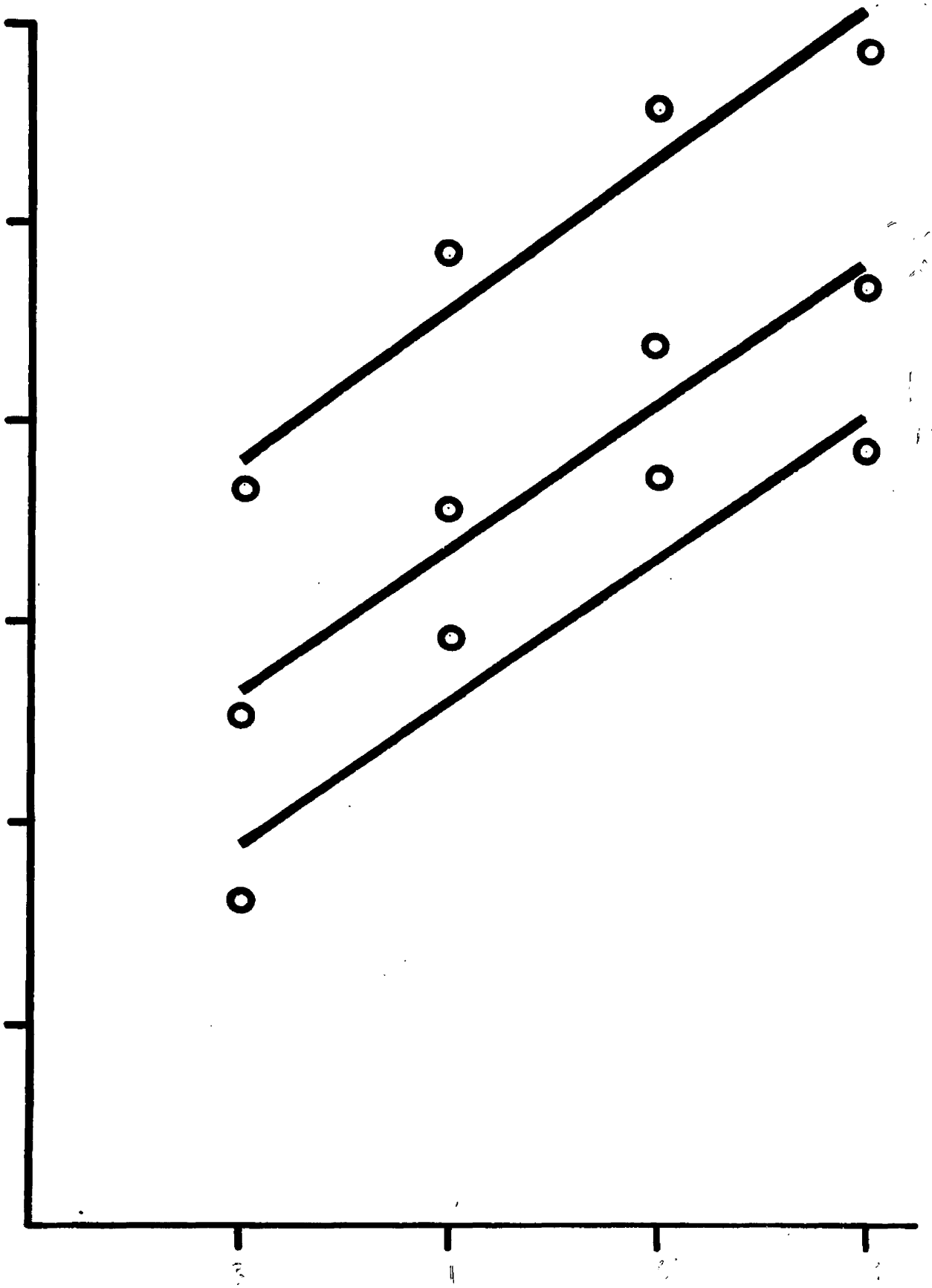
Cytochrome oxidase activity has previously been noted in the Leydig cells of foetal, prepubertal and adult mice of the Balb/c Jax strain (Hitzeman, 1962) and Sprague-Dawley rats (Niemi et al., 1962). The present findings differ from Hitzeman's experience in that no cytochrome oxidase can be detected prior to the end of the third week of postnatal life and this is probably attributable to strain variation. Of more consequence is the ease with which Hitzeman apparently demonstrated cytochrome oxidase which contrasts markedly with the great difficulty she encountered in visualising succinic dehydrogenase. In the present material, at any age after the third week, practically the entire interstitium contains demonstrable cytochrome oxidase activity while only a quarter or so of the Leydig cells possess succinic dehydrogenase activity in

histochemically detectable amounts. These facts suggest that there is a critical concentration of succinic dehydrogenase which must be reached before the Nachlas-Seligman reaction works and this would explain the absence of the enzyme from the Leydig cells of neonatal and immediately postnatal animals. In advancing this view the writer is aware that some of the apparent cytochrome oxidase activity is due to diffusion artefact but it seems unlikely that the large quantitative differences between the two enzymes can be adequately explained on this basis.

Mitochondrial morphology in the mouse Leydig cell does not differ significantly from the human morphology (Fawcett and Burgos,1960) but the inclusions described in woodchuck mitochondria (Christensen and Fawcett,1961) are absent. Fawcett and Burgos (1960) have described arcs and loops of the innermost limiting membrane in man and, while these occur in the mouse, they are rare and their significance obscure.

A comparison of Figs. 7 and 8 indicates that the three constituents of the Leydig cell studied namely its cytochrome oxidase, its succinic dehydrogenase and its mitochondria increase between birth and puberty and this increase is of a regular and progressive nature and is greatest during the fourth,fifth and sixth weeks of the

Figure 9. The Log graphs denoting the mitochondrial volume and the volume of Leydig tissue with succinic dehydrogenase and cytochrome oxidase are approximately parallel, indicating similar growth rates of the three fractions.



animals postnatal life. While the graphs contained in these figures suggest a general relationship between the three constituents it is not until one comes to study the log graphs (Fig.9) that one realises how close this liaison is. The gradients of the log graphs are indices of the rate of increase of the three constituents and the graphs are more or less parallel, each constituent increasing at approximately 12% per day during the period of maximal growth. This is very similar to the growth rate described for the Leydig tissue itself (Baillie,1961) and this implies that the complement of mitochondria, succinic dehydrogenase and cytochrome oxidase per Leydig cell is constant during the growing period. The steady and regular increase of the constituents studied thus merely reflects the progressive growth in volume of the Leydig tissue with age which in its turn determines androgen production.

SUMMARY

The succinic dehydrogenase and cytochrome oxidase content of the Leydig cell were studied by histochemical means in the mouse during the first ten weeks of postnatal life; ultrastructural details of Leydig mitochondria were examined at the same time.

Both enzymes appear at the end of the third week; mitochondria are present at all ages and do not change with advancing maturity. All three constituents increase with age, the increase is most marked during the 4th - 6th weeks of life, and all increase at a compound rate of about 12% per day. The three components are closely related, though some succinic dehydrogenase may have an extra-mitochondrial locus; their increase is thought to be merely an expression of Leydig tissue growth and does not represent a true rise in cellular or mitochondrial enzyme concentrations.

ACKNOWLEDGMENT

The author is grateful for the research facilities provided in the Anatomy Department of the University of Glasgow.

Baillie, A.H.: Observations on the growth and histochemistry of the Leydig tissue in the postnatal prepubertal mouse testis. J. Anat. Lond. 95, 357 - 370, 1961.

Baillie, A.H. and Griffiths, K.: Evidence for the existence of more than one 3β-hydroxysteroid dehydrogenase in the Leydig cells of the mouse testis. J. Endocrin: In press.

Burstone, M.S.: New histochemical techniques for the demonstration of tissue oxidase (cytochrome oxidase). J. Histochem. Cytochem. 7, 112 - 122, 1959.

Chatterjee, P., De, R. and Mitra, S.: Nucleolar localisation of succinic dehydrogenase. J. Histochem. Cytochem. 10, 6 - 7, 1962.

Chayes, F.: The theory of thin section analysis. J. Geol. 62, 92 - 101, 1949.

Christensen, A.K. and Fawcett, D.W.: The normal fine structure of opossum testicular interstitial cells. J. Biophys. Biochem. Cytol. 9, 653 - 670, 1961.

Conklin, J.L., Dewey, M.M. and Kahn, R.H.: Cytochemical localisation of certain oxidative enzymes. Amer. J. Anat. 110, 19 - 26, 1962.

Fawcett, D.W. and Burgos, M.H.: Studies on the fine structure of the mammalian testis II. The human interstitial tissue. Amer. J. Anat. 107, 245 - 270, 1960.

Glagolver, A.A.: Quantitative analysis by the "point method". Eng. and Mining J. 135, 399 - 400, 1934.

Hally, A.D.: Personal communication. 1963.

Hitzeman, Str. J.W.: Development of enzyme activity in the Leydig cells of the mouse testis. Anat. Rec. 143, 351 - 362, 1962.

Hooker, C.W.: The biology of the interstitial cells of the testis. Recent Progr. Hormone Res. 3, 173 - 196, 1948.

Lindner, H.R. and Mann, R.: Relationship between the content of androgenic steroids in the testes and the secretory activity of the seminal vesicles in the bull. J. Endocrin. 21, 341 - 360, 1960.

Luft, J.H.: Improvement in epoxy embedding methods. J. biophys. biochem. Cytol. 9, 409 - 414, 1961.

Moog, E.: Cytochrome oxidase in early chick embryos. J. cell. comp. Physiol. 22, 223 - 231, 1943.

Nachlas, M.M., Tsou, K.C., de Souza, E., Cheng, C.S. and Seligman, A.M.: Cytochemical demonstration of succinic dehydrogenase by the use of a new p-Nitrophenyl substituted ditetrazole. J. Histochem. Cytochem. 5, 420 - 435, 1957.

Nachlas, M.M., Crawford, D.T., Goldstein, T.P. and Seligman, A.M.: The histochemical demonstration of cytochrome oxidase with a new reagent for the Nadi reaction. J. Histochem. Cytochem. 6, 445 - 456, 1958.

Nieni, M. and Ekonon, N.: Cytochemistry of oxidative enzyme systems in the Leydig cells of the rat testis and their functional significance. Endocrinology, 70, 167 - 174, 1962.

Nieni, M. and Ekonon, N.: Histochemistry of the Leydig cells in the postnatal prepubertal testis of the rat. Endocrinology, 72, 443 - 448, 1963.

Ogawa, K. and Shinomaga, Y.: Intramitochondrial localisation of oxidative enzymes in cultured cells. J. Histochem. Cytochem. 10, 104 - 105, 1962.

Wallace, B.J.: The relation of mitochondrial morphology to succinoxidase activity as observed in the rat kidney after protein injection. J. Histochem. Cytochem. 8, 105 - 112, 1960.

Zetterqvist, H.: The ultrastructural organisation of the columnar absorbing cells of the mouse jejunum. Karolinska Institute, Stockholm, 1956.

HISTOCHEMICAL DIFFERENTIATION OF THE BASEMENT MEMBRANE OF THE MOUSE SEMINIFEROUS TUBULE.

Quart. J. micr. Sci. (1962), 103, 3, pp. 385-391.

SUMMARY

The ground substance of the testis of the albino mouse is PAS-positive but not metachromatic, and probably highly aggregated. The basement of the seminiferous tubules is intensely PAS-positive, metachromatic and possibly not so highly aggregated.

The reactivity of the ground substance to the PAS reaction and toluidine blue is tentatively ascribed to the presence of chondroitin sulphate C: this compound, previously known to contain N acetyl-galactosamine, glucuronic acid, tyrosine and tryptophane, is associated with arginine.

The genesis of the basement membrane of the seminiferous tubule is shown to include the formation of a sheath of atypical elongated fibroblasts, the secretion of a PAS positive, metachromatic substance associated with arginine between this sheath and the seminiferous tubule, the appearance of mitochondria in the cells of the sheath, and lastly, the acquisition of alkaline phosphatase by these fibroblasts and its spread to the intervening ground substance. These changes are thought to be related to the structural and nutritional requirements of the seminiferous tubules.

In its intense positive reaction to PAS and in its metachromasy, the basement membrane of the seminiferous

tubule agrees with the ground substance adjacent to sites of active protein metabolism, such as growing tumours, embryonic organs, hair follicles, and skin.

INTRODUCTION

Intercellular substances of the intertubular spaces of the testis, as in other connective tissues, fall into two principal groups, namely fibrillar structures and ground substance. The former comprise collagen, elastic, and reticular fibrils, which are thought to fulfil a largely mechanical function and whose distribution is well known. The ground substance consists of various colloids, crystalloids, gases, and water and is optically homogeneous when viewed with the light microscope. At a submicroscopic level it shows structural organization into a two-phase system (Gersh and Catchpole,1960) and the terms 'colloid-rich, water-poor phase' and 'colloid-poor, water-rich phase' have been coined by these workers in an attempt to describe it as a physico-chemical system. The colloids are known to include hyaluronic acid, chondroitin sulphates A,B and C, keratosulphate, and heparitin sulphate (Meyer, 1950): the water acts as a vehicle for ions, enzymes, hormones, vitamins, amino-acids, immune bodies, and protein originating from the plasma. The phases of this heterogeneous colloidal system are in electrical and

chemical equilibrium with one another. It is thought that the sol-gel consistency of the ground substance depends on the relative amounts of colloid and water present and this in turn is known to be influenced by fibroblastic activity, depolymerizing enzymes, hormones, growth, ageing, and other physiological and pathological processes (Gersh and Catchpole,1949).

The basement membrane may be defined as the region of specialized ground substance which intervenes between an epithelial structure and the ordinary ground substance. The present work is an account of the development of the basement membrane of the seminiferous tubule in the mouse testis between birth and puberty as revealed by various histochemical methods. From a functional point of view, any such description must include a consideration of the sheath of attenuated fibroblasts that surrounds the seminiferous tubule.

Material and methods

Thirty-six male Swiss White mice were used in preparation of the age series. The animals were killed in groups of 4 at weekly intervals between birth and the end of the eighth week of extra-uterine life. Two testes from each age group were fixed in a mixture of 90 ml water, 10 ml formalin, and 5 g mercuric chloride; in formaldehyde-calcium solution (90 ml water, 10 ml formalin, 1 g

anhydrous calcium chloride); in Helly's fluid; and in cold 70% ethanol.

The testes fixed in the first-named solution were dehydrated in cellosolve, embedded in estax (Watford Chemical Co.), and sectioned at 5 μ, a method found to preserve testicular morphology well (Baillie, 1960a). Sections were stained with haematoxylin and eosin, the McManus (1956) periodic acid/Schiff (PAS) technique (with methanol/chloroform and diastase controls), toluidine blue, methyl green, and Pyronin B for DNA and RNA, and also by a modified Sakaguchi reaction (Thomas, 1950) to show arginine. In addition, these stains were also controlled by digestion for 24 h with hyaluronidase (B.D.H.), buffered at pH 6.

Testes fixed briefly in formaldehyde-calcium solutions were embedded in gelatin and sectioned at 10 μ on the freezing microtome. Some sections were coloured with Sudan black to demonstrate the total lipids present; others were subjected to Hayes's (1949) modification of Feulgen and Voit's true plasmal reaction to show acetal phosphatides and possibly atypical -ketols (Boscott and others, 1948). Bennett's (1940) reaction was also employed on frozen sectioned material. 2.4-dinitrophenyl hydrazine wasused, notwithstanding the author's experience of this reagent (Baillie,1959).

Specimens fixed in Helly's fluid were sectioned at 4 μ and stained with toluidine blue and acid fuchsin to demonstrate mitochondria. Material fixed in 70% ethanol was sectioned at 5 μ in wax: alkaline phosphatase was demonstrated by a modified Gomori technique (Lillie, 1954).

RESULTS

Haematoxylin and eosin. At birth the seminiferous tubules are surrounded by an incomplete sheath of spindle-shaped mesenchymal cells, which have large, oval nuclei: the limits of the cytoplasm are clearly defined. Round some tubules the sheath of mesenchymal cells is complete; its nuclei are becoming elongated and stain more densely, and the cytoplasm is becoming attenuated and closely applied to the wall of the seminiferous tubule (fig.1,A). Eosinophil intercellular material is not present at this age. At the end of the first week of life the sheath-cells resemble atypical fibroblasts having greatly attenuated cytoplasm. Their nuclei, when seen in profile, appear as densely staining rods. Eosinophil material has appeared in the intertubular extracellular spaces and also between the sheath cells and the seminiferous tubules. For this reason cell boundaries are indistinct. This staining method does not reveal any further changes in the connective tissue of more mature testes. Hyaluronidase

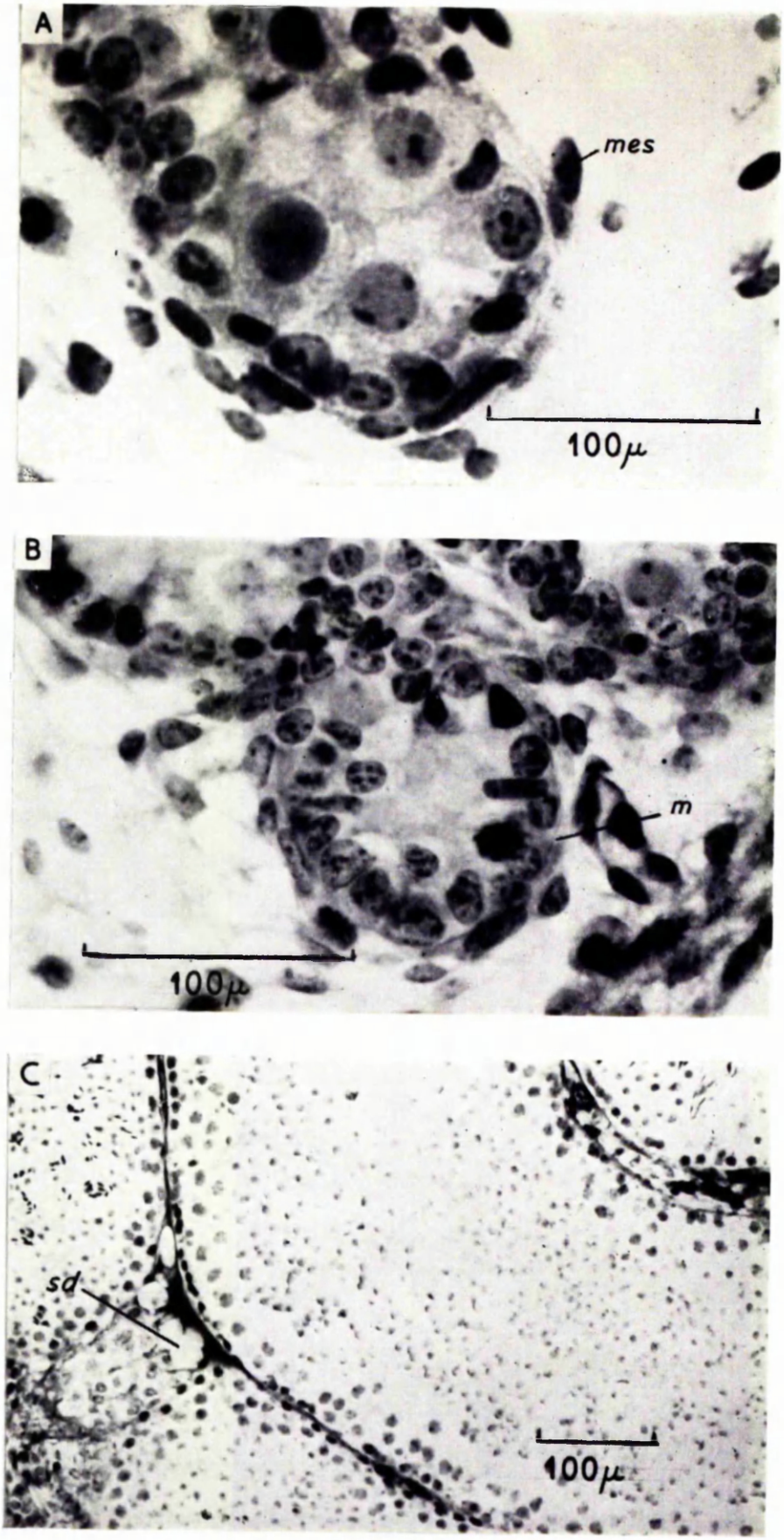


FIG. 1

FIG. 1 (plate). A, neonatal testis; H and E. Mesenchymal cells (*mes*) are condensing to form a sheath for the seminiferous tubule.

B, neonatal testis; PAS and haematoxylin. A fine membrane (*m*), reactive to PAS, is just visible in places round one seminiferous tubule.

C, testis, 4 weeks old; PAS and haematoxylin. The basement membrane of the tubule is prominent: staining defects (*sd*) are visible in the intertubular ground substance at one point.

has no effect on this picture.

The PAS reaction. At birth the intertubular extracellular spaces contain no PAS-reactive material. While the majority of seminiferous tubules have no PAS-positive basement membrane, there is a fine PAS-positive membrane beneath the mesenchymal sheath of the tubules, surrounded by a complete layer of fibroblasts (fig. 1,B). At the end of the first week the PAS-positive membrane, though exceedingly thin, is constantly present in the form of a red, refractile line surrounding all the seminiferous tubules. The extracellular spaces at this stage contain traces of PAS-positive material. With increasing age the PAS-positive basement membrane becomes slightly broader, and abundant PAS-positive ground substance becomes visible: the general ground substance does not stain so intensely with PAS as does the basement membrane of the tubules. Large staining defects occur at places in the ground substance (fig. 1,C); these resemble cartilage lacunae in shape and size but do not contain interstitial cells. Extraction with chloroformmethanol and diastase completely abolishes the PAS reactivity of the basement of the seminiferous tubules and also the reactivity of the general ground substance (fig. 2,A).

Toluidine blue. In the neonatal testis, beneath the mesenchymal sheath of the seminiferous tubules and

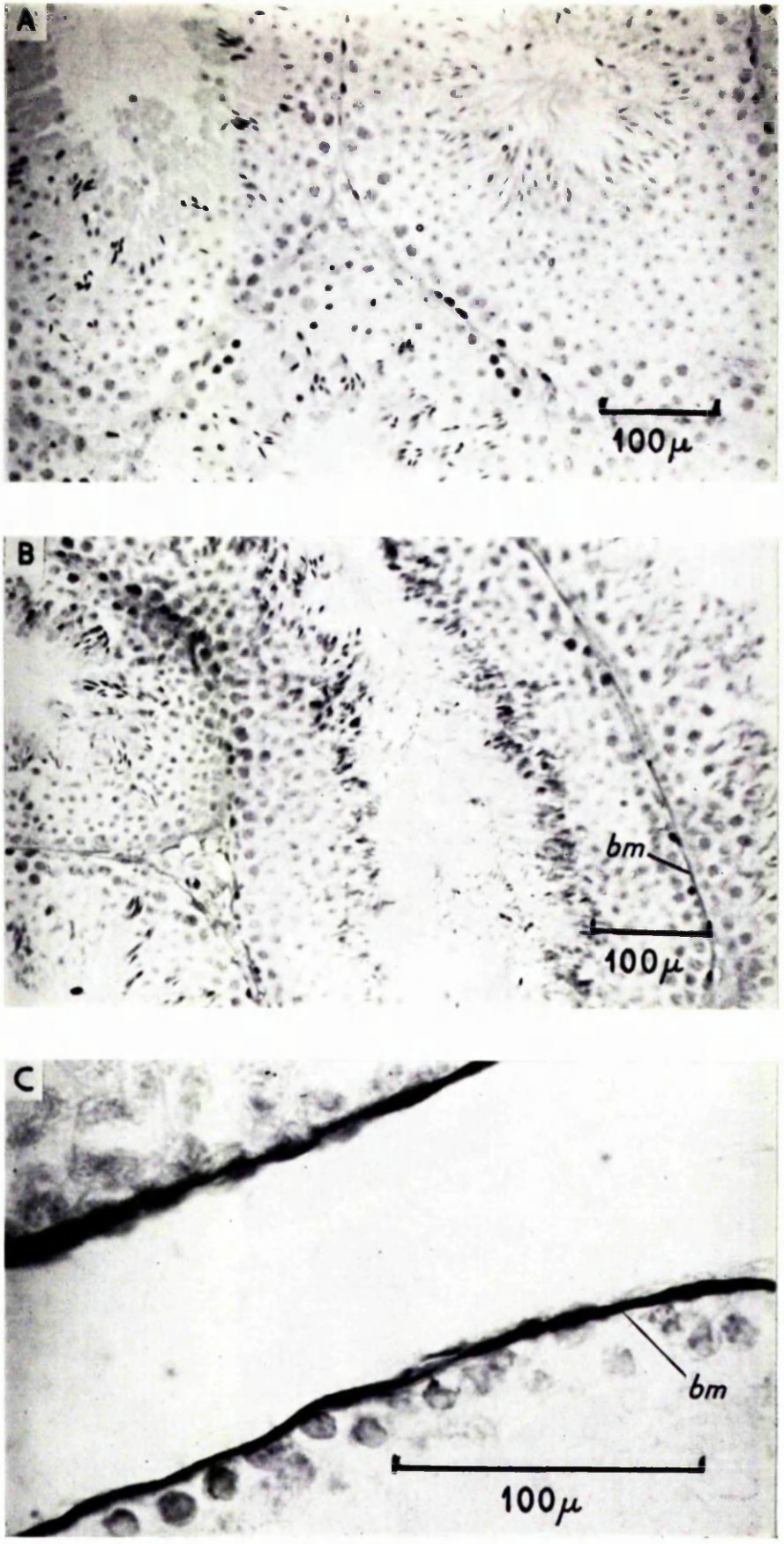


FIG. 2

FIG. 2 (plate). A, testis, 4 weeks old, after hyaluronidase digestion; PAS and haematoxylin. The reactivity of the basement membrane and ground substance to PAS has been abolished by enzymic hydrolysis.

B, testis, 5 weeks old; alkaline α-naphthol. The basement membrane (*bm*) of the seminiferous tubules contains arginine.

C, testis, 6 weeks old; alkaline phosphatase. The basement membrane (*bm*) of the tubule contains much alkaline phosphatase.

surrounded by a complete layer of cells there is a very fine membrane which stains metachromatically with toluidine blue. This membrane is present in all older testes. The general ground substance of the neonatal testis does not stain at all with toluidine blue, while the ground substance of older testes does stain, but not metachromatically. Hyaluronidase extraction removes the metachromatic properties of the basement membrane and diminishes the affinity of the general ground substance for toluidine blue.

Methyl green and pyronin B. The PAS-positive basement membrane and ground substance, whose distribution is described above, show a general weak affinity for these stains, giving a pale greenish-pink result. This affinity is reduced by hyaluronidase digestion but not by extraction with perchloric acid. This staining method reveals no other features of note.

-naphthol. The distribution of material demonstrated by this reagent closely parallels the reactivity of the ground substance and basement membrane to PAS. Thus at birth a few seminiferous tubules have a basement membrane containing arginine lying beneath a layer of fibroblasts. The seminiferous tubules of all older testes possess some arginine in their basement membranes (fig. 2,B). The extracellular spaces in the neonatal

testicular interstitium are devoid of material stainable with alkaline -naphthol. Later testes have extracellular material which stains faintly with alkaline -naphthol; it is distributed in the same manner as the PAS-positive material. Hyaluronidase extraction removes those components of the basement membrane and ground substance that stain with alkaline -naphthol, but has no effect on the affinity of spermatozoa and other cells for this reagent.

Sudan black. At no time between birth and puberty does the basement membrane of the seminiferous tubules or the general ground substance colour with Sudan black. The fibroblasts which ensheath the tubules contain a few sudanophil droplets.

The plasmal reaction. The neonatal testis is devoid of intercellular materials stainable with this reaction. The ground substance and basement membranes of the tubules in later testes stain pale pink with Schiff's reagent after oxidation with mercuric chloride. This coloration is not removed by acetone.

2.4-dinitrophenyl hydrazine. This reagent and the plasmal reaction appear to be staining the same structures between the tubules.

Mitochondria. At birth the mesenchymal cells surrounding the seminiferous tubules possess few mitochondria. Mitochondria are plentiful in these

fibroblasts at the end of the first week and in all subsequent testes; they take the form of minute granules which are located in small groups in the cytoplasm at each end of the nucleus.

Alkaline phosphatase. There is no alkaline phosphatase demonstrable by the Gomori method in the basement membrane of the seminiferous tubules during the first 14 days of extra-uterine life. Twenty-one days after birth the rod-shaped nuclei of the fibroblast sheath possess demonstrable alkaline phosphatase. All older testes are characterized by a black line, indicative of enzymatic activity, bounding the seminiferous tubules (fig. 2,C). Closer inspection suggests that much of this enzymatic activity is located in the PAS-positive basement membrane which intervenes between the fibroblasts and the seminiferous tubules, although some occurs in the nuclei and cytoplasm of the fibrocytes. In interpreting these findings it must be remembered that negative results with the Gomori phosphatase/cobalt sulphide method after alcoholic fixation and paraffin embedding are of doubtful significance, and that alkaline phosphatase in nuclei is widely regarded as an artifact.

DISCUSSION AND CONCLUSIONS

From the foregoing description it is apparent that the intercellular material of the testicular connective tissue conforms to Gersh's (1951) definition of ground substance in that it is optically homogeneous when viewed with the light microscope, gives a positive PAS-reaction, and (in places) stains metachromatically with toluidine blue. For a time it was widely believed that the ground substance matrix was a colloidal carbohydrate/protein complex which was aggregated to a variable extent and it was held that the amount of metachromasia and the intensity of the PAS-reaction exhibited were in inverse proportion to the degree of aggregation of the colloid. Recent studies (Gersh and Catchpole,1960) indicate, however, that the staining propensities of basement membranes are due to a preponderance of the 'colloid-rich, water-poor phase' in a very finely corrugated layer of ground substance.

The central difficulty in assessing the present findings lies in the conflict of opinion regarding the interpretation to be placed on the results of the staining methods used. Meyer (1950) claimed that metachromatic, PAS-positive substances fall into five categories, namely, hyaluronic acid, hyaluronic acid monoester sulphate, and the 3 chondroitin sulphates, A,B, and C; but several recent workers have suggested that the metachromatic and the PAS-

positive components of tissues are naturally separate, or can be separated by experimental manipulations. Thus, Einbinder and Schubert (1951) showed that pure chondroitin sulphate, which is a strong chromotrope, reduces periodate only very slowly. Further, Glegg and others (1954), by differential alcoholic precipitation of alkaline tissue-extracts, separated PAS-positive fractions of several tissues from metachromatic fractions. Moreover, Braunstein and Buerger (1959) made a clear separation in vitro of metachromatic material from PAS-staining material in amyloid.

It has been established (Keyer,1950) that hyaluronidase hydrolyses hyaluronic acid, hyaluronic acid monoester sulphate, and two of the chondroitin sulphates, chondroitin sulphate B being resistant to enzymic extraction. Since hyaluronidase abolishes the PAS-reactivity of the seminiferous tubular basement membrane and intertubular ground substance, it may be concluded that the PAS-positive material is probably an acid mucopolysaccharide and that chondroitin sulphate B is not present in the testis in histochemically demonstrable amounts, since it would have survived hyaluronidase extraction. Chondroitin sulphate A may also be excluded as the basis of the PAS-reactivity of the testicular intertubular ground substance, since this mucopolysaccharide is probably peculiar to hyaline cartilage. Further, it is generally considered that aqueous fixation and

an aqueous PAS method, such as that employed in the present investigation, do not preserve hyaluronic acid and hyaluronic acid monosulphate (Lillie,1954). Hotchkiss (1948) used alcoholic solutions and obtained different results. These observations suggest that chondroitin sulphate C is probably the PAS-reactive component of the basement membrane of the mouse seminiferous tubule, but it is difficult to reconcile this with Leblond's (1957) statement that acid mucopolysaccharides fixed with chromate do not give the PAS reaction under the usual histochemical conditions (that is, with brief periodic acid oxidation). Possibly the formaldehyde/mercuric chloride fixation employed in the present study increases the reactivity of acid mucopolysaccharides to PAS. Alternatively, hyaluronidase may digest Leblond's (1957) heteropolysaccharides in addition to acid mucopolysaccharides.

The above observations indicate that the genesis of the basement membrane of the seminiferous tubules involves a number of stages which may be arbitrarily distinguished from one another. First, indifferent mesenchymal cells metamorphose into atypical elongated, ensheathing fibroblasts with attenuated cytoplasm and densely staining, rod-shaped nuclei. Secondly, a PAS-positive substance, associated with arginine and metachromatic material, appears between the

cytoplasm of the seminiferous tubule and that of the fibroblast sheath. While this complex is being elaborated mitochondria appear in the cytoplasm of the sheath fibroblasts. Lastly, alkaline phosphatase seems to appear in the nuclei of these cells, and maturation is completed by the spread of this enzyme to the cytoplasm of the sheath fibroblasts and also to the ground substance of the basement membrane.

These changes in the mouse are largely completed by the end of the animal's fourth week of extra-uterine life, the time at which spermatozoa appear in the tubules. Clearly the structural changes are related to the increased support requirements of the growing tubule, and the mitochondrial changes possibly reflect fibroblastic synthesis of the polysaccharide complex which forms the PAS-positive membrane. The enzymic changes may parallel the increase in the nutritional requirements of the tubule, particularly glucose. While the majority of the seminiferous tubules in the mouse acquire their PAS-positive membranes after birth, the seminiferous tubules of the sheep have well defined PAS-positive basement membranes long before birth (Baillie, 1960b).

At the periphery of invasive tumours and in rapidly growing embryonic organs the ground substance of the related connective tissue becomes intensely metachromatic

and PAS-positive during the phase of active growth (Gersh , 1951). A comparable phenomenon has been described in the ground substance of the dermal papilla of an actively growing hair follicle and also in the connective tissues involved in the repair of dermal damage (Montagna,1956). In each of these various sites, cessation of growth is followed by progressive aggregation of the carbohydrate-protein complexes, with the concomitant loss of metachromasia and PAS reactivity. Similarly, the basement membrane of growing and functioning seminiferous tubules is highly PAS-positive and metachromatic, and this suggests by analogy with the foregoing that the degree of aggregation of connective tissue mucopolysaccharides is a generalized reflection of active protein metabolism.

From the Sudan black, plasmal, and 2.4-dinitrophenyl hydrazine tests it is clear that free neutral fats, acetal phosphatides, and atypical -ketols (Boscott and others, 1948) are not present in the basement membrane with the plasmal test and 2.4-dinitrophenyl hydrazine, with resistance to acetone extraction, may possibly be a weak pseudoplasmal reaction of the type described by Cain (1949) and attributable to aldehydes known to be present in the elastic fibres of rodents (Pearse,1960).

The author is grateful for the research facilities provided in the Anatomy Department of Glasgow University.

REFERENCES

BAILLIE, A.H., 1959. 'Endeavour' Essay, British Association.

_____ 1960a. J. Endocrin., 20, 339.

_____ 1960b. Quart. J. micr. Sci., 101, 475.

BENNET, H.S., 1940. Amer. J. Anat., 67, 151.

BOSCOTT, R.J., MANDL, A.M., DANIELLI, J.F., and SHOPPEE, C.W., 1948. Nature, 162, 572.

BRAUNSTAIN, H., and BUERGER, L., 1959. Amer.J.Path., 35,791.

EINBINDER, J., and SCHUBERT, M., 1951. J. biol. Chem., 191, 591.

GERSH, I., 1951. Connective Tissues, 2, 11.

_____ and CATCHPOLE, H.R., 1949. Amer. J. Anat., 85, 457.

_____ _____ 1960. Perspectives in Biology and Medicine, 3, 282.

GLEGG, R.E., EIDINGER, D., and LEBLOND, C.P., 1954. Science, 120, 839.

HAYES, E.R., 1949. Stain Tech., 24, 19.

HOTCHKISS, R.D., 1948. Arch. Biochem., 16, 131.

LEBLOND, C.P., GLEGG, R.E., and EIDINGER, D., 1957. J. Histochem. Cytochem., 5, 445.

LILLIE, R.D., 1954. Histopathologic technic and practical histochemistry. New York (Country Life Press).

McMANUS, J.F.A., 1956. Nature, 178, 914.

MEYER, K., 1950. Connective Tissues, 1, 88.

MONTAGNA, W., 1956. The structure and function of skin. New York (Academic Press).

PEARSE, A.G., 1960. Histochemistry, theoretical and applied. London (Churchill).

THOMAS, L.E., 1950. Stain Tech., 25, 143.

VALLANCE-OWEN, J., 1948. J. Path. Bact., 60, 325.

ULTRASTRUCTURAL DIFFERENTIATION OF THE BASEMENT MEMBRANE OF THE MOUSE SEMINIFEROUS TUBULE

by

A.H. BAILLIE.

(From the Department of Anatomy, The University, Glasgow).

Quart. J. micr. Sci. In Press.

SUMMARY

The development of the basement membrane of the mouse seminiferous tubule has been studied between birth and the end of the tenth week of postnatal life using the electron microscope. The sheath cells which surround the seminiferous tubules are derived from mesenchymal cells, and differentiation of these specialised cells continues for five weeks after birth. Between the plasma membranes of the sheath cells and those of the Sertoli cells there are three distinct zones. The zone of decreased electron density nearest the seminiferous tubule is thought to be specialised extracellular fluid filtered by the second or intermediate zone which consists of numerous fine particles, and the term pseudomembrane is advanced for this zone. Collagen develops in the broad outer zone next to the sheath cells and fulfils the support requirements of the growing tubule.

INTRODUCTION.

Using the electron microscope (Bennett, 1963) most cells have been shown to possess a mucopolysaccharide coating closely applied to the lipoprotein plasma membrane of Danielli. The erythrocyte is a notable exception (Adams, 1960) but it elicits immunological responses which

can only be satisfactorily explained by the existence of such a coat (Kabat, 1956). The continuity of this mucopolysaccharide coat over the surface of contiguous epithelial cells gives rise to an electron dense layer which corresponds roughly with sites of PAS reactivity in light microscope sections and is the basement membrane of electron microscopists. Yamada (1955) has shown that it consists of many fine interlacing filaments about 90Å in diameter, and the spaces between these filaments may be the basis of the filter action attributed by Farquhar & Palade (1960) to this membrane.

In a previous communication to this journal (Baillie, 1962) the basement membrane of the mouse seminiferous tubule was found to be a layer of PAS positive, metachromatic material lying between the tubule epithelium and a surrounding sheath of atypical fibroblasts, and it was thought to contain chondroitin sulphate and arginine. The maturation of this membrane was marked by its acquisition of alkaline phosphatase.

This paper represents an attempt to follow the development of the basement membrane of the mouse seminiferous tubule with the electron microscope, and sets out to correlate the results with the known histochemical facts and current theories of basement membrane

ultrastructure.

MATERIAL AND METHODS.

Eight male Swiss White mice were killed singly aged 0, 7, 14, 21, 28, 35, 42, and 70 days. The neonatal testes were fixed intact while teased preparations of the older gonads were used. After 30 minutes fixationin 1% buffered osmic acid (Zetterqvist, 1956) the material was dehydrated in methanol and embedded in araldite (Luft, 1961). Thin sections cut on Huxley and Porter-Blum microtomes were mounted on uncoated copper grids and stained in 1% methanolic lead acetate. The specimens were screened in a Philips electron microscope EM.75B at 60 Kv. with original magnifications of 1,100 to 11,000.

RESULTS.

At birth sections of the testis (Fig. 1A) show irregular stellate or spindle shaped mesenchymal cells in the process of becoming applied to the periphery of the seminiferous tubules. They have elongated nuclei with no features of note. The cytoplasm contains scattered, usually elongated, mitochondria with tubular cristae together with a few irregular osmiophilic inclusions, probably lipid in nature; scattered RNA particles and a disseminated Golgi apparatus are also present. The cell

membrane has no features of note.

At low magnifications (Fig. 1B) the seminiferous tubules appear to be bounded by a double membrane inside the mesenchymal sheath, but closer inspection at higher magnifications (Fig. 1C) indicates that the peripheral cells of the seminiferous tubule have only a solitary membrane, the plasma membrane, and applied to its external surface is a band of extermely fine, moderately electron dense, particles. It is this outer dense lamina of fine particles which at lower magnifications simulates a membrane. Between this band of material and the plasma membrane of the tubule cell is an area of less electron dense material at this age. A similar broader area intervenes between this band of electron dense material and the sheath cell plasma membrane. No fibrillar structures are present, and none of the plasma membranes adjacent to the intercellular space presents any features of special interest.

The seminiferous tubules of the seven day old mice have a complete investment of sheath cells one layer thick and these are becoming more flattened and closely applied to the surface of the tubule. No other changes occur in the first week.

By the end of the second week the entire seminiferous

FIGURE 1 (plate).

(A) Neonatal testis. Spindle shaped mesenchymal cells (M) are in the process of becoming flattened on the surface of the seminiferous tubule (ST).

(B) At low magnifications the neonatal seminiferous tubules (ST) appear to have a double membrane (DM).

(C) At higher magnifications the "double membrane" is seen to resolve into an inner plasma membrane (PM) derived from the Sertoli cell (S) and an outer, moderately electron dense pseudomembrane (P). An elongated mitochondrion (MC) is seen in the adjacent sheath cell.

FIGURE 2 (plate).

(A) The nuclei (N) of overlapping sheath cells (SC) are seen in the fourteen day old testis.

(B) An occasional mesenchymal cell (M) becoming applied to the sheath cell layer of the 14 day old animal.

(C) The pseudomembrane (P) of the fourteen day old mouse is separated from the plasma membrane (PM) of the Sertoli cell (S) by a layer of moderately electron dense material (IZ). A larger zone (OZ) intervenes between the pseudomembrane and the sheath cell (SC) and contains large particles of unknown nature. Osmiophilic lipid droplets (L) occur in the sheath cell cytoplasm.

(D) Collagen fibres (C) appear in the outer zone (OZ) of the basement membrane of the four week old mouse.

Fig. 1.

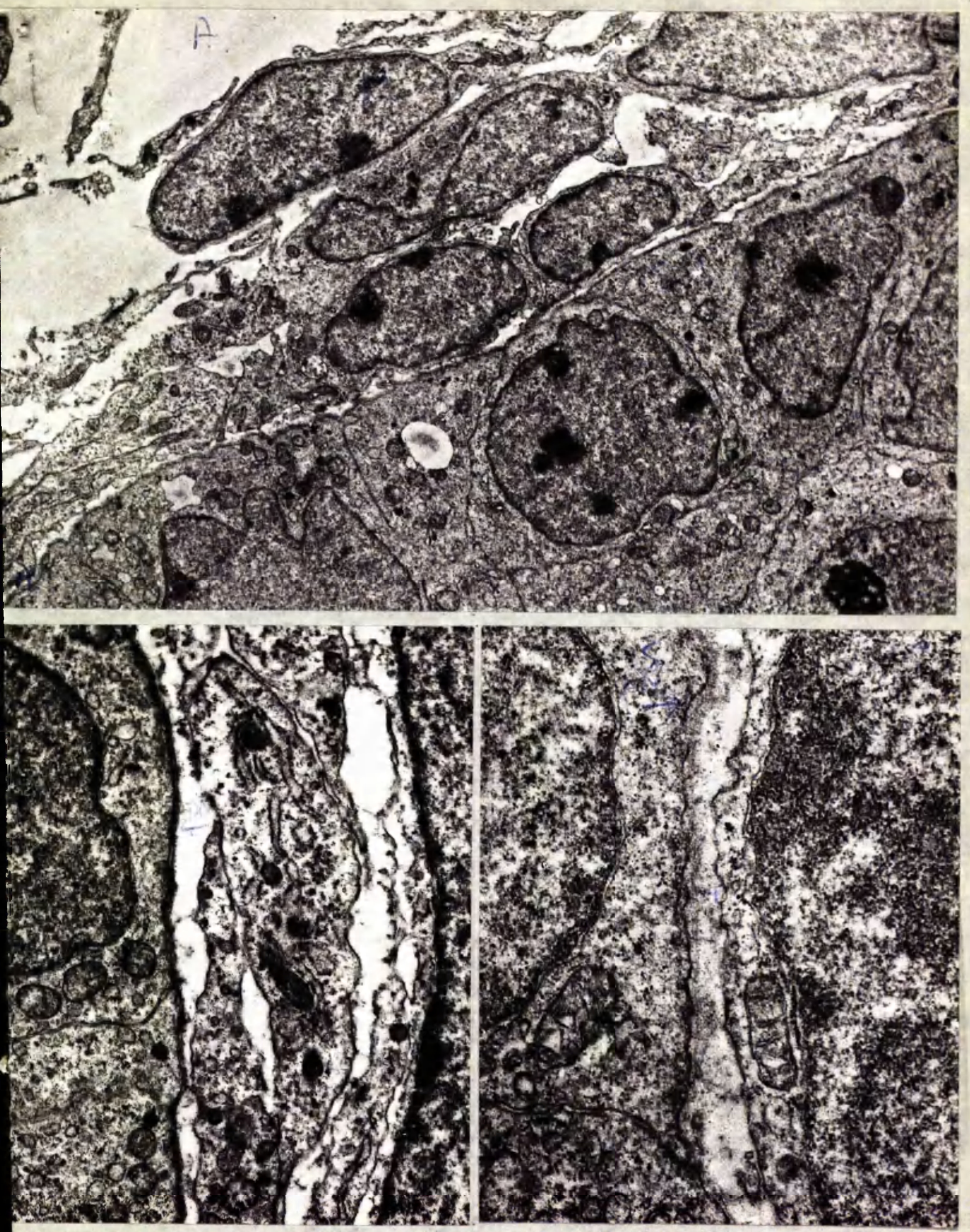
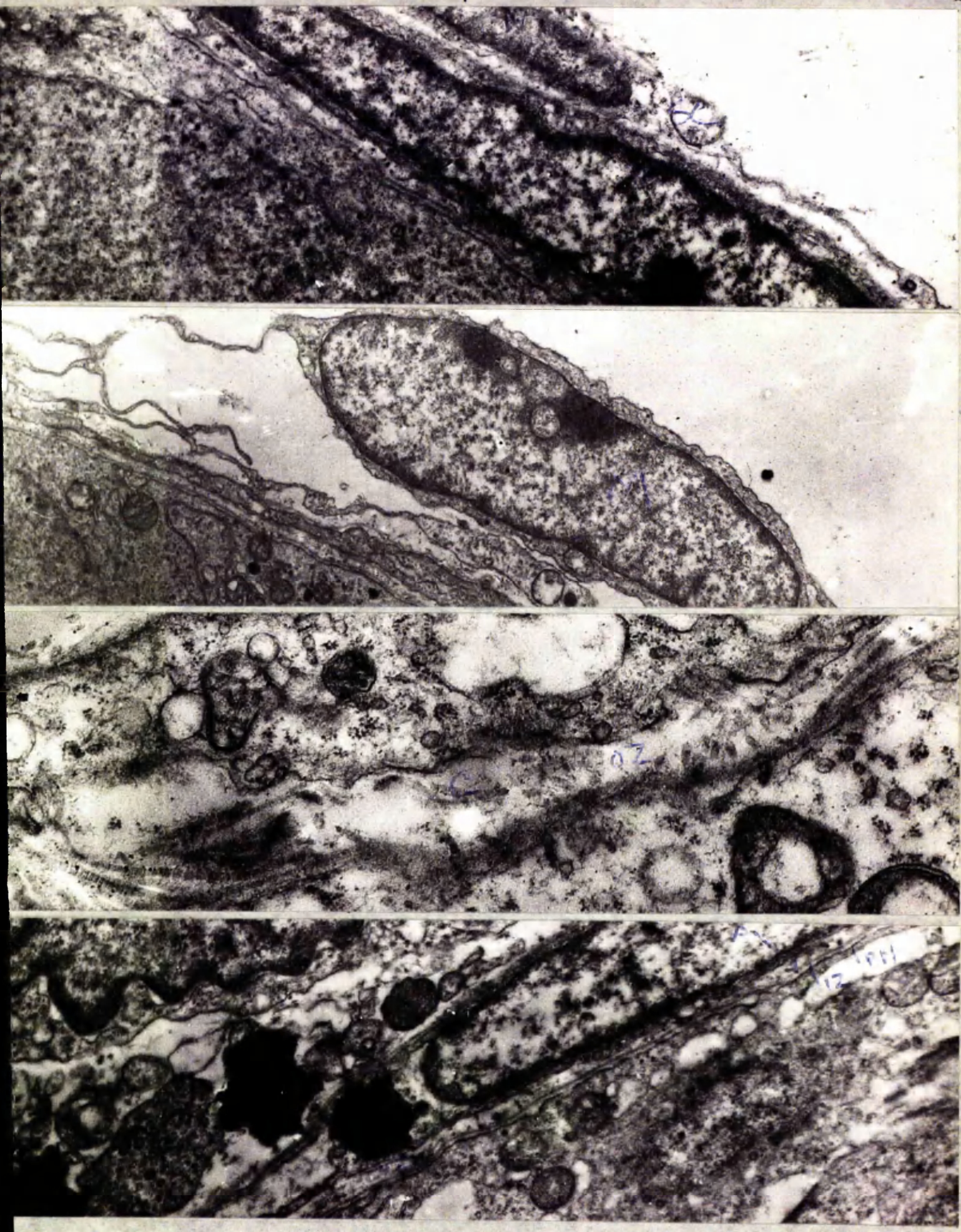


Fig. 2.



A

B

D

C

tubule is closely surrounded by flattened sheath cells with elongated nuclei and these cells overlap one another to a marked extent in some places (Fig. 2A) and the seminiferous tubule may be invested by the thin cytoplasmic processes of three or more cells in some places. The mitochondria of sheath cells have become more rounded and osmiophilic lipid droplets are still to be seen, becoming flattened and applied to the tubules (Fig. 2B).

The interval between the plasma membrane of the sheath cell and that of the outermost cells of the seminiferous tubule is divided into three recognisable regions (Fig. 2C):-

(1) Immediately outside the plasma membranes of the peripheral seminiferous tubule cells is a zone of moderate electron density with no organised structure in it. The plasma membranes of the Sertoli cells have occasional caveolae opening into this space.

(2) A zone of higher electron density which is thicker and less definite than a plasma membrane delimits the outer aspect of zone (1). This pseudomembrane corresponds in electron density characteristics with the outer dense lamina described in the neonatal testis and is apparently the same structure.

(3) Between the pseudomembrane and the plasma membrane of

the sheath cell is a zone which is much broader than the previous two zones, less electron dense than either, and which contains a number of particles of variable electron density. These particles are on the whole rather larger than RNA particles, and some of the larger ones may be collagen.

With the continuing growth of the tubules, the sheath cells become more and more attenuated, and by the end of the third week of postnatal life mitochondrial diameter has come to exceed the thickness of its containing cytoplasmic process, with the result that mitochondria come to form projections on the surface of the cell directed away from the seminiferous tubule. Otherwise, the fourteen and twenty-one day old basememt membrane regions are identical.

During the fourth week of life many changes of note occur. RNA particles become a prominent inclusion of the sheath cell cytoplasm and large irregular cytoplasmic vacuoles appear (Fig. 2D). The mitochondria assume rather bizarre shapes and the sheath cell plasma membrane develops many indentations and becomes difficult to recognise in places. Much the most significant feature at this stage is the appearance of large numbers of collagen fibres on both sides of the sheath cell. The fibres appearing between the sheath cells and the tubules are restricted

to the outermost of the three zones described in the fourteen day old animal and have no special orientation. The three zones persist, but the boundaries between one and two are rather less definite.

The 35, 42, and 70 day old animals are very similar: the collagen fibres tend to become orientated so that they are arranged perpendicularly to the seminiferous tubules. A second, sparser group of fibres at right angles to these appears and persists into adult life.

DISCUSSION.

When the present electron microscope findings are compared with the corresponding light microscope results (Baillie, 1962), many similarities and a few apparent differences are encountered. In both studies the formation of the seminiferous tubule basement membrane includes the laying down of a sheath of atypical fibroblast-like cells round the tubule, and this is largely complete by the end of the first week of life. The electron microscope has shown in addition, however, that as the tubules grow the sheath cells elongate markedly and the degree of overlap between adjacent sheath cells is steadily reduced. The progressive addition of indifferent mesenchymal cells to the sheath as the animal matures was also not noted in

the light microscope study. In both studies the sheath cells were seen to contain mitochondria, but the juxtanuclear disposition of these organelles seen under the light microscope has not been confirmed with the electron microscope. The significance of this is not clear, but perhaps the different methods of preparation of the two groups of specimens affects the final apparent distribution of these organelles. Occasional sudanophilic lipid particles were seen in the cytoplasm of the sheath cells at all ages in the previous study, and these correspond in site and size with the irregular osmiophilic inclusions seen under the electron microscope. The vacuoles revealed by the electron microscope in the sheath cells when collagen is first produced have no light microscope equivalent. The RNA particles, which are so abundant in the sheath cells at the end of the fourth week, are probably a reflection of the collagen synthesis by these cells at this time.

The significance of the three layers found between the plasma membranes of the seminiferous tubule cells on the one hand and the sheath cells on the other, is more difficult to interpret. The zone of decreased density nearest to the seminiferous tubule has previously been described (Bennett, 1963) and is thought by that writer to be an area of specialised extracellular fluid, filtered

by the electron dense pseudomembrane or glycocalyx and destined for absorption by pinocytosis. Only occasional structures resembling the caveolae of other workers were seen in the present study.

The intermediate zone of electron dense material seen surrounding the mouse seminiferous tubules probably accounts for the PAS reactivity and methachromasia of the tubule basement membrane when viewed under the light microscope. This is in keeping with the correlation between PAS reactivity and extracellular electron dense material shown to exist at other sites, for example, muscle sarcolemma (Schriebler, 1953; Hama, 1960) amoeba cell wall (Brandt & Pappas, 1960) and renal glomerulus (Farquhar & Palade, 1960; Yamada, 1955). In relation to the plasma membranes of the Sertoli cells no desmosomes were noted. The outer dense zone or pseudomembrane noted in the present study has a fine particulate appearance which is quite different from the filamentous nature of the basement membranes of capillaries (Yamada, 1955) and this may be due to the different physiological activities of basement membranes in different sites.

The appearance of single collagen fibrils in the outer zone immediately adjacent to the sheath cell plasma membrane probably reflects the support requirements of the

growing tubule and may account for the reticular fibres seen using the light microscope.

The author is grateful for the research facilities provided in the Anatomy Department of Glasgow University.

REFERENCES.

ADAMS, C.D., 1960. Anat. Rec., 136, 151.

BAILLIE, A.H., 1962. Quart. J. micr. Sci., 103, 385.

BENNETT, H.S., 1963. J. Histochem. Cytochem., 11, 14.

BRANDT, P.W. and PAPPAS, G.D., 1960. J. Biophys. Biochem. Cytol., 8, 675.

FARQUHAR, M.G., and PALADE, G.E., 1960. J. Biophys. Biochem. Cytol., 7, 297.

HAMA, K., 1960. J. Biophys. Biochem. Cytol., 7, 717.

KABAT, E.A., 1956. Blood Group Substance: Their Chemistry and Immunochemistry. Academic Press, New York.

LUFT, J.H., 1961. J. Biophys. Biochem. Cytol., 9, 409.

SCHIEBLER, T.H., 1953. Z. f. Zellforsch., 39, 152.

YAMADA, E., 1955. J. Biophys. Biochem. Cytol., 1, 445.

ZETTERQVIST, H. 1956. The Ultrastructural organisation of the columnar absorbing cells of the mouse jejunum. Karolinska Institutet, Stockholm.

THE BIOLOGY OF THE LEYDIG CELL: HISTOCHEMICAL AND HISTOLOGICAL CHANGES FOLLOWING HIGH EPIDIDYMAL OBSTRUCTION.

J. Endocrin. (1960), 20, 339-344.

SUMMARY

Fourteen albino male mice were subjected to bilateral section and ligation of the epididymis at a point just distal to the proximal lobules of the head, and were killed 2,4,8,12,16,20 and 24 weeks after operation. One testis was studied using the periodic acid-Schiff (PAS) technique, the other by frozen sections. The weight of the seminal vesicle was taken as an indicator of androgen output.

Testicular histology remained unchanged during the 6 months after operation, and there was no alteration in the weight or cytology of the seminal vesicles. An attempt is made to relate the findings to current views on Leydig cell biology.

The postoperative occurrence of abdominal Leydig cells is described: these cells contain abundant PAS-positive cytoplasmic material (?glycoprotein) and have not previously been noted. Their anatomical and physiological significance is not clear.

The effects of ligation, infarction and removal of portions of the excurrent duct system of the testis (vasa efferentia, epididymis, vasa deferentia) have been the object of intensive study since Bouin & Ancel (1903) claimed that obstruction of the vas deferens was followed by atrophy of the seminiferous tubules and hypertrophy or hyperplasia of the interstitial elements and Leydig cells. The literature pertaining to such experiments is copious and contradictory. Most recent workers agree, however, that in the rat at least, obstruction of the vasa efferentia or of the initial segment of the epididymal head, which lies immediately distal to the terminal vas efferens, whether by ligation or infarction, is followed by degeneration of the seminiferous epithelium and an apparent increase in the total volume of Leydig cells present (MacMillan, 1952; Harrison, 1953a,b). On the other hand, occlusion of the tail of the epididymis or of the vas deferens is thought to be without effect on the testis (Moore, 1932).

On the basis of these conclusions it was decided to study, by histochemical means, the changes taking place in the Leydig tissue following epididymal obstruction at a point immediately distal to the

proximal lobules of the caput epididymidis and to attempt to relate such changes to alterations in the animal's internal endocrine environment, as indicated by fluctuations in the size and cytology of the seminal vesicles.

MATERIAL AND METHODS

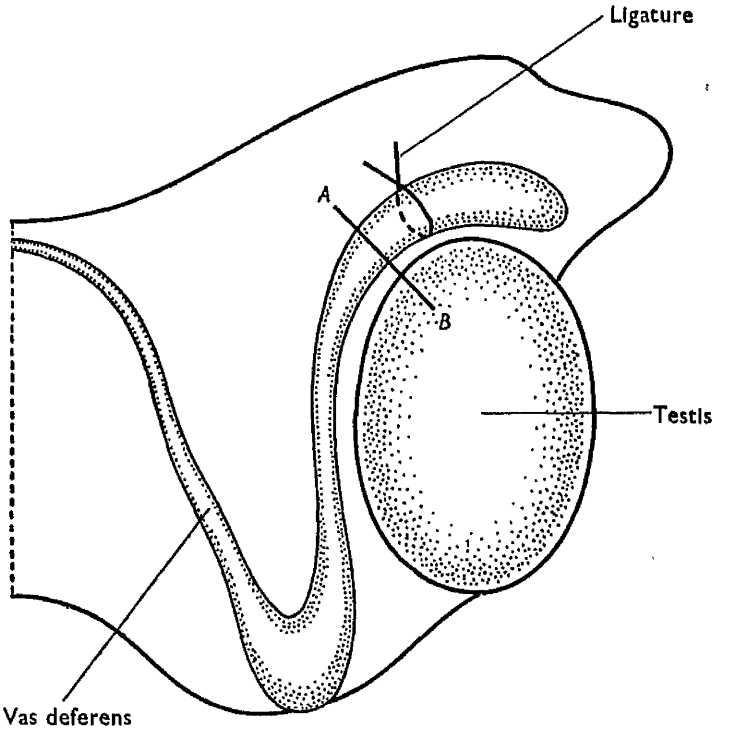
Six adult male albino mice were killed: their seminal vesicles were removed and fixed in formol corrosive solution (90 ml. water, 10 ml. formalin, 5 g mercuric chloride). Forceps were used to occlude the outlet of the vesicles during the initial stages of fixation to prevent loss of secretion by agonal contraction. The vesicles were then weighed, dehydrated, embedded in ester wax, sectioned at 5 μ and stained with haematoxylin and eosin. An epididymis from each animal was similarly treated. One testis from each animal was processed in the same fashion, some sections being stained by the McManus/Hotchkiss periodic acid-Schiff (PAS) procedure (Carleton & Drury, 1957) and others with haematoxylin and eosin. The other testis was fixed in formol-calcium solution (90 ml. water, 10 ml. formalin, 1 g calcium chloride): it was then embedded in gelatin and sectioned at 10 μ on the freezing microtome. Some sections were stained in Sudan black to demonstrate total lipids present, others

were subjected to Hayes' modification of Feulgen and Voit's plasmal reaction (Lillie,1954) to show acetal phosphatides and possibly steroids (Dempsey,1948). Testicular volume was determined on this testis using the formula:

$$V = \frac{4}{3}\pi b^2 a,$$

where V = testicular volume, b = half the minor axis of the testis, and a = half the major axis. This established controls for the project.

The fourteen animals used in the main experiment were killed in pairs 2,4,8,12,16,20 and 24 weeks after operation. The testes of the operated animals were delivered, under surgical anaesthesia, through bilateral aseptic incisions in the anterior abdominal wall. The caput epididymidis was transected at the site shown on Text-fig.1 and the end proximal to the testis was ligated; care was taken not to injure the regional blood vessels. The vasa efferentia, the initial segment and the proximal lobules of the head of the epididymis were left patent and in communication with the seminiferous tubules. The gonads were returned to the abdomen and the wounds sewn up in the customary fashion. When the animals were subsequently killed the epididymides were inspected to ensure that no ligatures had been displaced. What remained of the



Text-fig. 1. Site of transection of caput epididymidis (A—B).

proximal part of the epididymis was fixed in formol corrosive, sectioned, and either stained with haematoxylin and eosin or subjected to Perl's Prussian blue reaction (Carleton & Drury, 1957). The testes and seminal vesicles from the experimental animals were treated in the same way as those from the control animals.

RESULTS

(1) Seminal vesicle

Throughout the 6-month period covered by the experiment no macroscopic changes, such as loss of turgor, were detected in the seminal vesicles. There was no significant deviation in the weight of the vesicles of operated animals from the adult control mean weight of 240 g (Table 1), nor were there any histological changes visible during this time.

(2) Testis

Volume. Minor fluctuations from the control mean of 199 mm^3 were noted (Table 1); these, however, were not regarded as significant, since all observations fell within the normal limits of variation established.

Microscopic structure. The histological picture of the testis obtained by routine staining with haematoxylin and eosin was constant throughout the period under consideration. Spermatogenesis continued normally, even 24 weeks after epididymal obstruction, and there was no

Table 1. *Mean weight of seminal vesicle and of volume of testis after varying periods of epididymal obstruction*

Duration of obstruction (weeks)	0	2	4	8	12	16	20	24
Weight of seminal vesicle (mg)	240	250	245	220	235	250	250	235
Testicular volume (mm^3)	199	199	193	185	197	200	198	192

evidence of tubular atrophy or dysfunction (Plate, fig.1). The Leydig cells retained their characteristic adult form, being hexagonal in shape and having abundant cytoplasm filled with minute pale yellow refractile granules. Their nuclei were typically ovoid, vesicular and possessed a characteristic chromatin arrangement; mitotic figures were not observed nor were the Leydig cells more prominent than usual at any time.

(a) PAS-stained material. The vast majority of Leydig cells present a characteristic appearance when subjected to the PAS procedure; the abundant foamy cytoplasm stains a pale pink colour and a few cells contain bright red, diastase resistant, refractile granules. The nuclei contain no PAS-positive material. The extracellular spaces, not readily seen in the corresponding material stained with haematoxylin and eosin, are well defined and contain a PAS-positive polysaccharide complex. In the testes taken from operated animals some Leydig cells contain large PAS-reactive granules, dispersed through the cytoplasm. These are connected, by intermediate forms, with abnormally large rounded Leydig cells whose cytoplasm is packed full of PAS-positive granules. These peculiar cells have from one to five densely staining nuclei (Plate, figs.2,3). They, in their turn, appear

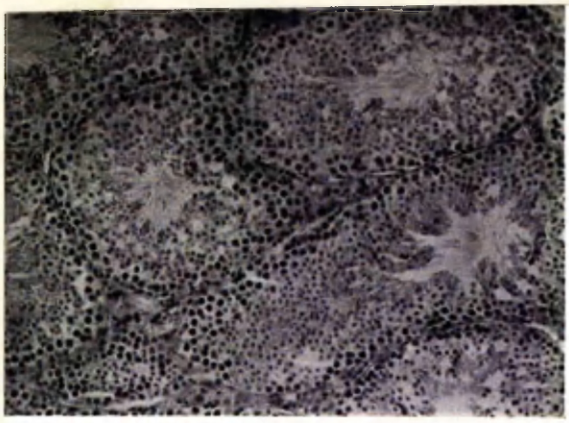


Fig. 1

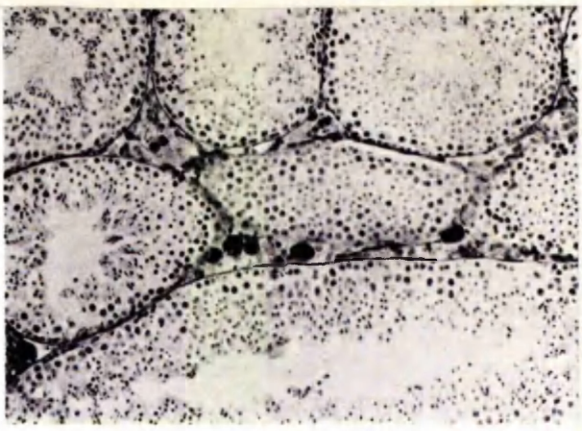


Fig. 2

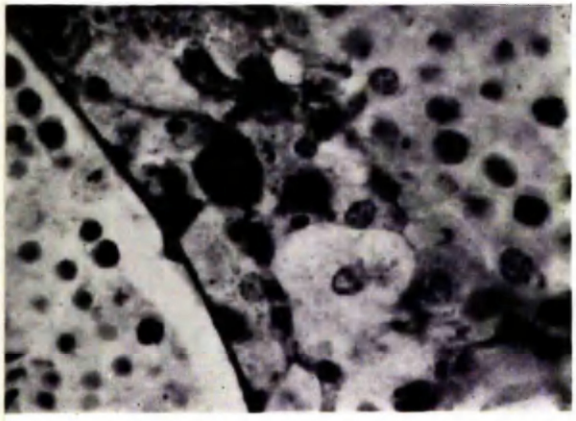


Fig. 3

Fig. 4

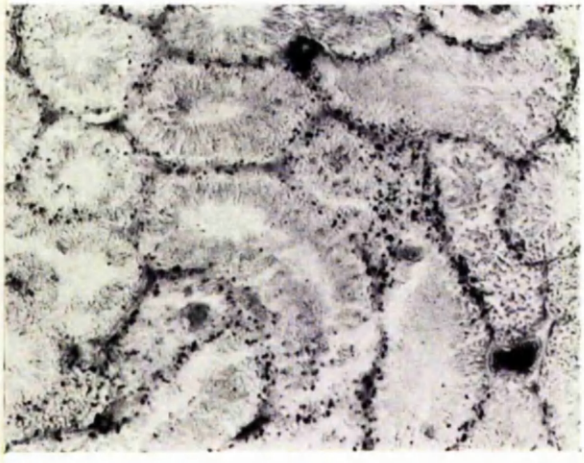


Fig. 5

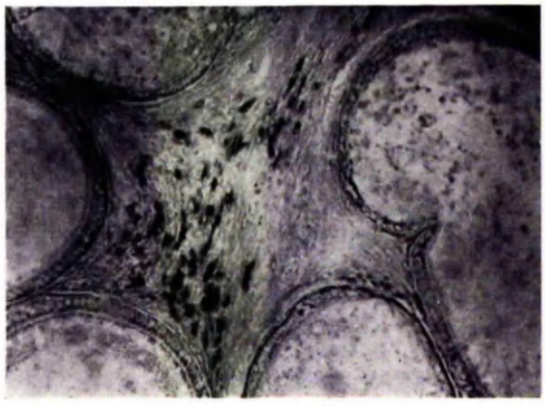


Fig. 6

DESCRIPTION OF PLATE

Fig. 1. Testis, 24 weeks after operation. Normal appearance of interstitium and continuation of spermatogenesis in the seminiferous tubules. (H. & E, × 150.)

Fig. 2. Testis, 2 weeks after operation. Presence of atypical Leydig cells characterized by variable amounts of PAS-positive material in the cytoplasm. (PAS, × 150.)

Fig. 3. Testis, 2 weeks after operation. Abnormal PAS-positive Leydig cells. (PAS, × 350.)

Fig. 4. Testis, 16 weeks after operation. No alteration in distribution of sudanophilic Leydig material following epididymal occlusion. (Sudan black, × 90.)

Fig. 5. Testis, adult control. Typical distribution of Leydig and Sertoli plasmalogens is unaltered by epididymal occlusion. (Plasmal reaction, × 90.)

Fig. 6. Head of epididymis, 12 weeks after operation. Tubules of initial segment contain numerous spermatozoa; fibrosis of intertubular tissue is advanced and many haemosiderin-containing macrophages are present. (Prussian blue reaction, × 150.)

to be linked, by transition forms, with small rounded cells, with PAS-positive granular cytoplasm and small, densely staining nuclei. The PAS-positive material to be found in these abnormal cell types resists digestion by diastase and solution in chloroform; the cells are prominent 2 weeks after operation, present but scarce 4 weeks after it, and virtually absent in all subsequent periods.

(b) Sudan black material. Control sections of adult testes stained with Sudan black present a uniform picture which is in no way affected by high epididymal obstruction (Plate, fig.4). The entire interstitium, with the exception of blood vessels and ground substance, is stained heavily. The individual Leydig cells are large polygonal or rounded elements with abundant lipid-laden cytoplasm. The lipid droplets seem to comprise large irregular masses formed by coalition of small sudanophilic granules, which occur singly at the cell periphery.

(c) The plasmal reaction. Control and experimental sections present the same appearance after this reaction (Plate, fig.5). Most of the Leydig cells stain a pale pink colour, which resists acetone decolorization and must, therefore, be regarded as a negative response. A few Leydig cells contain abundant deeply staining

material which dissolves in acetone and which is due to myriads of minute red staining granules. In the seminiferous tubules droplets of purple staining (PAS-positive) lipid are to be seen in the Sertoli elements.

Quantitative observations. In the mouse all mature Leydig cells are found to have abundant cytoplasmic lipids which stain readily with Sudan black. A large number of random sections of the testes at each time interval were stained in this fashion (Plate, fig.4) and projected on to paper, at a magnification of 1800 diameters, when the areas of black tissue (i.e. Leydig cells) were delineated by pen. Sections from all regions of the testis were examined. A figure expressing the Leydig tissue as a percentage of the entire organ was derived by weighing the total paper field and, later, the cut-out areas representing Leydig tissue. The accuracy of the method depends on adequate staining and the examination of a large number of sections by the same operator. Using this technique the total volume of Leydig tissue was found to remain constant at 4.5% of the volume of the testis throughout the 6 months after the operation: the volume of Leydig tissue containing PAS stainable lipids (acetal phosphatides and possibly steroids) was also estimated at each time

interval and remained constant at 0.8% of the testicular volume.

(3) Epididymis

During the postoperative period the residual caput epididymidis proximal to the testis becomes visibly distended: the diameter of the epididymal tubules increases some three- or fourfold and there is evidence of fibrous hyperplasia in the surrounding connective tissue. The tubules of the initial segment, which in the mouse are normally virtually devoid of spermatozoa, come to contain numerous spermatozoa (Plate, fig.6). These facts, taken together with the secure positioning of the ligatures at death, were regarded as indicative of the completeness of obstruction: further in the vicinity of the ligatures there are no visible patent tubules, only fibrous tissue and haemosiderin-containing phagocytes.

DISCUSSION

Since there was no significant alteration in seminal vesicle size or cytology during the postoperative period, androgen output is probably unchanged; this view is supported by the constancy of the testicular lipid distribution.

The significance of the abnormal Leydig cells, prominent during the first 4 weeks after operation, is

not clear. The carbohydrate material is neither glycogen nor glycolipid since it resists the action of diastase and the lipid solvents; its appearance cannot be related to any quantitative change in Leydig function, since seminal vesicle weight remains constant, nor is it associated with any visible upset of spermatogenesis. The occurrence of these peculiar aberrant Leydig cells is to be regarded as an incidental finding which may possibly be attributed to the trauma of surgical interference; further work is needed to clarify this point.

It has previously been shown (Moore, 1932) that obstruction of the vas deferens in the dog, cat, sheep, rabbit and man has no effect on spermatogenesis - a normal testis may even be found despite the complete congenital absence of the excurrent duct system. On the other hand, MacMillan (1952) finds that infarction of the initial segment of the caput epididymidis is followed by sloughing of the germinal epithelium and oedema of the interstitial tissue. Similar sequelae attend ligation and division of the vas efferentia (Harrison,1953a,b). It has been suggested by these workers that occlusion of the tail of the epididymis or of the vas deferens has no effect on the testis because the caput and body of the epididymis act as a distensible reservoir which

accommodates the spermatozoa in such a fashion as to avert any significant back pressure on the tubules of the testis; the tubular disruption and interstitial oedema which follow occlusion of the vasa efferentia or of the initial segment of the head have been attributed to the disruptive effects of back pressure.

The lack of any upset in the cytology of the seminiferous tubules in the present experiment was contrary to the author's expectations and may be due to the small residual portion of the epididymal duct system, which was left in communication with the seminiferous tubules acting as a distensible reservoir in the fashion suggested by Harrison and his co-workers. One wonders whether the residual caput epididymidis could compensate in such a manner for 6 months, particularly when sections of this portion of the epididymis suggest that maximal distension occurs within a few days of operation. However that may be, it seems clear that an obstruction of the mouse epididymis which leaves the proximal lobules of the head, the initial segment and the vasa efferentia in patent communication with the seminiferous tubules, is not followed by the intratesticular upsets which have been described as sequelae to occlusion of the vasa efferentia or of the initial segment of the caput epididymidis in the rat:

at the point chosen in this experiment spermatolysis in the duct system proximal to the ligature appears to offset testicular production of spermatozoa.

Nevertheless, it must be pointed out that a remarkable pattern of testicular disorganization follows many diverse experimental manipulations. Within a few weeks the entire germinal epithelium sloughs, leaving shrunken empty tubules lined by the Sertoli syncytium, while the interstitium becomes oedematous and Leydig hyperplasia may supervene. Such changes have been described variously as sequelae to ligation and division of the vasa efferentia (Harrison,1953a,b), infarction of the initial segment of the caput epididymis (MacMillan,1952), obstruction of the vas deferens (Bouin & Ancel,1903), occlusion of the testicular artery (Oettlé & Harrison, 1952) and also following ischaemia induced by immersing the gonad in freezing solutions at -3.5^{o} to $-5^{o}C$ (Harris & Harrison, 1955). The amazing similarity, both histological and chronological, of the changes which follow these various experimental procedures suggests to the author the possibility of some common underlying mechanism which initiates the above sequence of events. Clinical experience has shown that surgical treatment of varicocoele and of inguinal hernia may be complicated by testicular pain, testicular hardening and subsequent atrophy.

Moreover, Baker & Evoy (1942) have observed that in some cases even gentle surgical manipulation of tissues may be followed by marked vasoconstriction with at least temporary ischaemia of the part involved. It remains to be seen whether the site of epididymal obstruction is the only critical factor in the causation of testicular disruption.

The author is grateful for the research facilities provided at the Anatomy Department at the University of Glasgow.

REFERENCES

Baker, J.W. & Envoy,M.M.(1942). Surg. Gynec.Obstet. 75,285.

Bouin,P. & Ancel,P.(1903). Arch. zool.exp.gen. 1,437.

Carleton,H.M.& Drury,R.A.B.(1957). Histological technique, pp.143;162. Oxford University Press.

Dempsey,E.W.(1948). Recent Progr.Hormone Res. 3,127.

Harris,R. & Harrison,R.G.(1955). Proc.Soc.Study Fertil. 7,23.

Harrison,R.G.(1953a). Proc.Soc.Study Fertil. 5,97.

Harrison,R.G.(1953b). Proc.Soc.Study Fertil. 5,101.

Lillie,R.D.(1954). Histopathology technic and practical histochemistry, p.316. New York: Blakiston.

MacMillan,E.W.(1952). Proc.Soc.Study Fertil. 4,23.

Moore,C.R.(1932). In Sex and internal secretions,p.353. Ed. E. Allen. Baltimore: Williams and Wilkins.

Oettle,A.G. & Harrison,R.G.(1952). J. Path.Bact. 64,273.

OBSERVATIONS COMPARING THE EFFECTS OF EPIDIDYMAL OBSTRUCTION AT VARIOUS LEVELS ON THE MOUSE TESTIS WITH THOSE OF ISCHAEMIA.

J. Anat., Lond. (1962), 96, 3, pp 335-354.

INTRODUCTION

Previous experiments (Baillie, 1960) have failed to demonstrate any visible upset of spermatogenesis following bilateral ligation of the epididymis at a point just distal to the proximal lobules of the head. There is considerable evidence, however, that unilateral obstruction of the initial segment of the caput epididymidis (Harrison & Macmillan, 1954) or of the ductuli efferentes (Harrison, 1953) in the rat is followed by substantial testicular atrophy and interference with spermatogenesis. Obstruction of the cauda epididymidis in the rat, on the other hand, is without effect on the testis (Macmillan, 1953). It was, therefore, decided to undertake experiments in which unilateral epididymal obstruction was effected at various levels in an attempt to investigate the claim of Harrison and his co-workers that the caput and corpus epididymidis act as a distensible reservoir which may accommodate spermatozoa so averting significant back-pressure on the seminiferous tubules. These findings were compared with the effects of ischaemia on the mouse testis.

MATERIAL AND METHODS

The animals used were adult male Swiss white mice aged from 3 to 6 months and weighing from 45-60 g. One hundred and seventy mice were used in all, thirty being subjected to each of the following surgical procedures.

Operation no. 1. Unilateral ligation of the initial segment of the caput epididymidis at the level of the proximal lobules. This corresponds with the level of obstruction described by Harrison & Macmillan (1954) following infarction of the caput.

Operation no. 2. Unilateral ligation of the caput at a point immediately distal to the proximal lobules of the head. This is the level of obstruction previously described by the author (Baillie, 1960) following bilateral ligation.

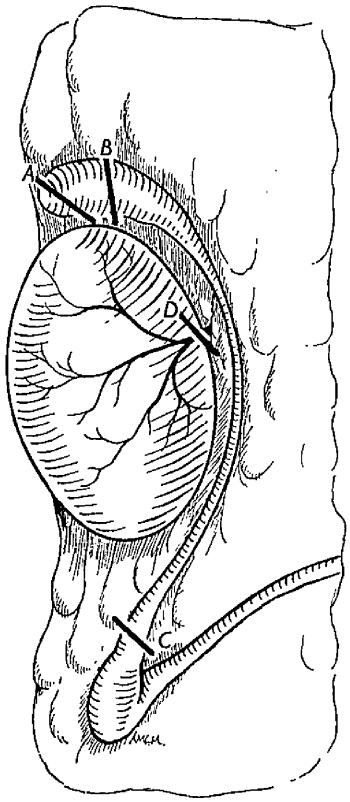
Operation no. 3. Unilateral ligation of the cauda epididymidis at a point which corresponds with the obstruction described by Macmillan (1953) following ligation of the vasal and inferior epididymal arteries.

Operation no. 4. Ligation of the testicular vascular pedicle, including the artery and veins, 3 mm. or so from the testis, an arrangement which leaves the blood supply of the epididymis intact.

Operation no. 5. A control operation in which one testis was exteriorized for 10 min. and subjected to manipulation with forceps before being returned to the abdomen. The other remained intact in situ.

In each instance the right testis was delivered under surgical anaesthesia through an aseptic right-sided incision. The left testis remained in situ as an intact control. Care was taken not to injure the blood vessels of the part. The operated gonad was returned to the abdomen on completion of the appropriate procedure and the wound sewn up in the usual manner. The various operative procedures are illustrated diagramatically in Text-fig. 1.

In the past, histological assessment of the sequelae of excurrent duct obstruction has been entirely dependent on subjective impressions of spermatogenic activity and no attempt has been made to measure cell turnover in the tubules. For this reason spermatogenic activity was estimated in terms of colchicine arrested mitoses. One animal from each group was killed daily to form a post-operative series covering 1 month. Each animal received an intraperitoneal injection of colchicine (0.1 mg. in water/100 g. body weight) at 10.00 a.m. on the appropriate day, 5 hr. before sacrifice. The period



Text-fig. 1. The three sites of epididymal obstruction are denoted by the letters *A*, *B*, and *C*. The testicular vascular pedicle was ligated at point *D*.

of colchicine administration chosen was identical with that used by Ebling (1954) and Bullough (1950) who selected it to vitiate the effects of the diurnal mitotic cycle.

The testes were excised, dissected free of mesorchium and weighed rapidly before being subjected to a modified McManus/Hotchkiss periodic acid-Schiff (PAS) reaction (Baillie,1959) which minimizes testicular shrinkage and distortion of Leydig tissue. The chemical specificity of this modified reaction is discussed elsewhere (Baillie, 1962). All tissues were processed in precisely the same manner: fixation in a mixture of 90 ml. water, 10 ml. formalin, and 5 g. mercuric chloride was undertaken within 2 min. of excision and lasted 6 hr. Dehydration in cellosolve was completed in 24 hr. when the tissues were impregnated for 3 hr. in ester wax. Controls employed included methanol-chloroform extraction, digestion with diastase and digestion with hyaluronidase (B.D.H.) buffered at pH 6 for 24 hr.

The mitotic rate was calculated by counting the total number of cells in the layer of germinal epithelium adjacent to the basement membrane in a large number of seminiferous tubules and also the number of basal cells in

the same tubules whose nuclei were in arrested mitosis: from these figures the mitotic rate is derived as a percentage per 24 hr. It must be pointed out, however, that this method of assessment of tubular mitotic rate is by no means free from fallacy. In particular, during the tubular expansion described below, the epithelium of the seminiferous tubules is thinned from something like six to three layers without loss of cells. In these circumstances it is impossible to be sure that none of the cells nearer the centre of the lumen move down to lie adjacent to the basement membrane during the thinning process. If such a movement does not occur then the germinal epithelium must multiply rapidly or be attenuated in a lateral direction to occupy the required area. It is technically not possible to perform accurate mitotic counts on the entire seminiferous epithelium of the enlarged tubules described below on account of their size and it was therefore felt that the mitotic rate of the basal layer of the cells of the seminiferous tubules was the best available mitotic index, provided its shortcomings are remembered.

In addition to the experimental animals, twenty normal mice of the same age group were given colchicine and their testes treated as outlined above in order to

establish the range of individual variation of tubular mitotic activity.

In the adult mouse (Baillie, 1959) all seminiferous tubules are bounded by a strongly PAS positive basement membrane. Twenty-five sections of testes from each animal were projected by microscope on to paper, when the tubules were delineated by pen. The relative masses of intertubular and tubular tissues were derived as a percentage by weighing the total paper field, and, later the cut out areas representing the seminiferous tubules. The total masses of the intertubular and tubular tissues were calculated from the percentage and the testicular weight.

The diameter of the seminiferous tubules was measured routinely by projection microscopy using a scale calibrated in 1/100 mm.

At sacrifice the epididymides were inspected to ensure that no ligatures had been displaced. What remained of the proximal part of the epididymis was sectioned and subjected to the PAS reaction and microscopic examination to ensure that epididymal obstruction was complete.

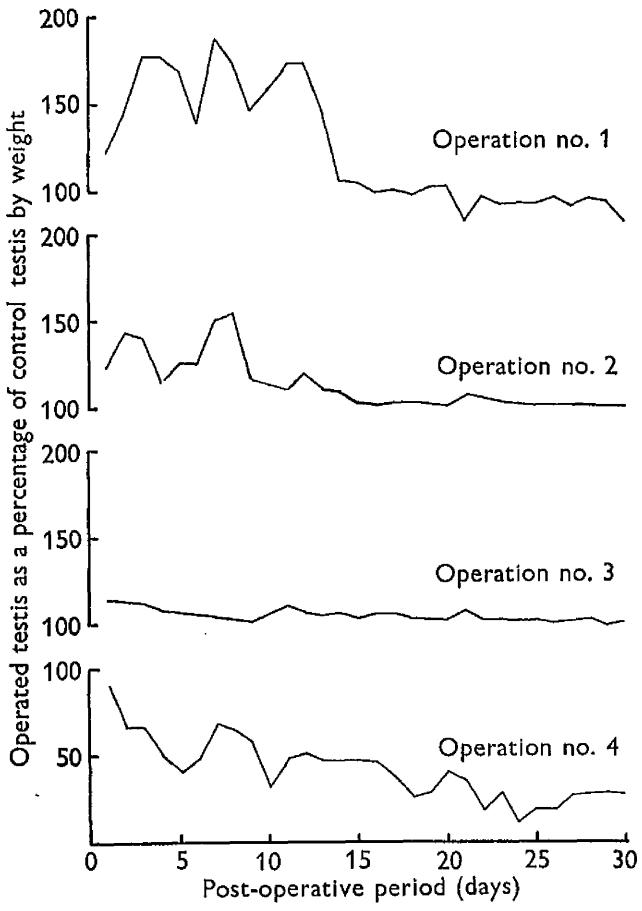
RESULTS

(1) Testicular weight

As will be seen from Table 1 and Text-fig. 2, following obstruction of the initial segment of the caput epididymidis

Table 1. *Mensural data of testes following operation*

	Operation no. 1			Operation no. 2			Operation no. 3			Operation no. 4		
Post-operative period (days)	Weight of control testis (mg.)	Weight of operated testis (mg.)	Operated testis as a % of control testis	Weight of control testis (mg.)	Weight of operated testis (mg.)	Operated testis as a % of control testis	Weight of control testis (mg.)	Weight of operated testis (mg.)	Operated testis as a % of control testis	Weight of control testis (mg.)	Weight of operated testis (mg.)	Operated testis as a % of control testis
1	195	243	124·7	172	214	124·5	187	215	115·0	194	168	86·6
2	186	269	144·6	204	294	144·1	231	261	113·0	215	142	66·1
3	164	291	177·4	215	301	140·1	194	219	112·9	183	121	66·1
4	181	320	176·8	187	216	115·5	164	179	109·2	209	109	52·2
5	194	329	169·6	208	264	126·9	204	222	108·8	222	93	41·9
6	183	253	138·2	216	271	125·4	203	216	106·4	196	97	49·5
7	179	336	187·7	180	272	151·1	200	211	105·5	191	132	69·1
8	205	356	173·5	218	338	155·0	195	202	103·6	185	121	65·4
9	207	301	145·4	190	222	116·9	209	213	102·0	200	119	59·5
10	211	335	158·8	194	222	114·5	222	237	106·8	231	77	33·4
11	207	361	174·4	192	214	111·4	192	215	111·9	201	100	49·8
12	222	385	173·4	216	262	121·3	215	232	107·9	198	102	51·5
13	191	278	145·5	200	213	106·5	213	226	106·1	222	110	49·6
14	221	233	105·4	201	212	105·4	203	217	106·9	188	91	48·4
15	197	197	100	195	201	103·0	217	225	103·7	185	90	48·6
16	195	185	94·9	204	208	102·0	222	234	105·4	191	89	46·6
17	215	206	95·8	200	209	104·5	190	200	105·2	197	78	39·6
18	195	184	94·4	185	194	104·8	195	204	104·6	190	49	25·8
19	232	227	97·8	196	202	103·0	181	187	103·3	213	64	30·0
20	220	217	98·7	210	216	102·9	210	219	104·2	181	78	43·1
21	198	154	77·8	201	219	108·9	182	196	107·6	198	72	36·4
22	234	226	96·6	180	193	107·3	210	215	102·3	198	39	19·7
23	188	177	94·1	175	182	104·0	177	183	103·4	201	58	28·9
24	234	222	94·9	205	212	103·4	247	253	102·4	191	21	11·0
25	221	209	94·6	210	212	100·9	173	180	104·1	188	38	20·2
26	179	174	97·2	186	190	102·2	209	214	102·4	210	42	20·0
27	235	217	92·3	215	221	102·8	210	218	103·9	235	69	29·4
28	189	183	96·8	202	203	100·5	215	225	104·7	179	53	29·6
29	179	170	95·0	220	223	101·4	212	212	100	215	64	29·8
30	200	166	83·0	204	207	101·5	199	200	100·5	213	61	28·6



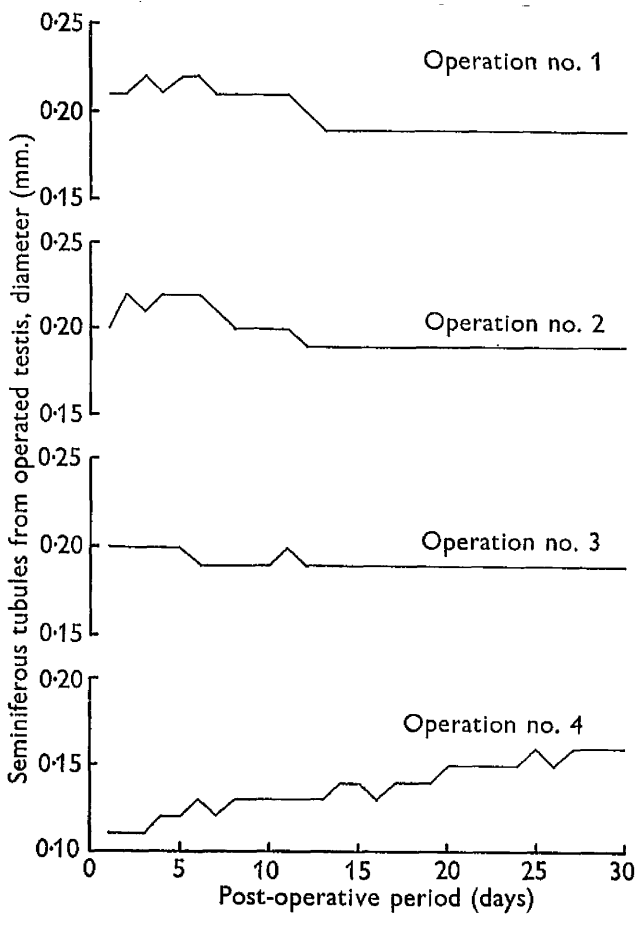
Text-fig. 2. The testis responds to epididymal obstruction by a phase of initital weight gain, the magnitude of which increases as the site of obstruction moves proximally to the testis: this phase lasts 12 days and is followed by a period of slight persistent weight gain in the case of the two lower obstructions, and by a period of slight weight loss in the case of obstruction of the initial segment. Infarction is followed by progressive weight loss.

(operation no. 1) the operated testis expands immediately and abruptly by 77.4% within 3 days; further individual maxima of 87.7 and 74.4% are reached on the seventh and eleventh days, respectively. Since only one mouse per day has been studied it is not possible to be sure whether these peaks indicate true or individual variation: with a larger series the responses depicted in Text-fig. 2 might well be plateau like. This increase in size is a transient phenomenon and terminates suddenly between the 12th and the 14th day. The remarkable testicular enlargement during what may be termed the phase of initial weight gain is illustrated in Pl. 1, fig. 1. Between the 15th and the 30th days the operated testis exhibits a degree of slight atrophy of the order of 5 or 7%, with individual extremes of 22.2% on day 21 and 17% on day 30.

After obstruction immediately distal to the proximal lobules of the head (operation no.2), the operated testis shows a similar sudden weight gain of more moderate dimensions (Text-fig. 2 and Table 1) with individual maxima of 44.1, 55.0 and 21.3% on the 2nd, 8th and 12th days, respectively. As before, between the 12th and the 14th day this increase in weight is lost rapidly but, in marked contrast with the foregoing, not completely. A slight weight gain of 1-4% persists during the second half of the period surveyed.

Table 2. *Mensural data of seminiferous tubule diameter after operation, together with mitotic rates of the germinal epithelium*

Post-operative period (days)	Tubule diameter in control testis (mm.)	Tubule diameter in operated testis (mm.)	Mitotic rate in control testis (%)	Mitotic rate in operated testis (%)	Tubule diameter in control testis (mm.)	Tubule diameter in operated testis (mm.)	Mitotic rate in control testis (%)	Mitotic rate in operated testis (%)
	Operation no. 1				Operation no. 2			
1	0·19	0·21	12·9	8·1	0·19	0·20	14·1	9·2
2	0·19	0·21	12·1	9·2	0·19	0·22	12·7	9·1
3	0·19	0·22	11·6	8·7	0·19	0·21	10·9	8·6
4	0·19	0·21	9·5	9·0	0·19	0·22	10·0	8·8
5	0·19	0·22	9·7	9·6	0·19	0·22	8·9	9·1
6	0·19	0·22	8·1	8·4	0·19	0·22	8·5	9·0
7	0·19	0·21	7·3	7·0	0·19	0·21	7·1	7·0
8	0·19	0·21	6·9	7·2	0·19	0·20	7·3	7·1
9	0·19	0·21	7·2	6·9	0·19	0·20	6·8	7·0
10	0·19	0·21	7·0	6·8	0·19	0·20	6·9	6·7
11	0·19	0·21	6·4	6·5	0·19	0·20	6·9	6·9
12	0·19	0·20	7·3	6·6	0·19	0·19	6·8	6·9
13	0·19	0·19	6·9	6·6	0·19	0·19	6·6	7·0
14	0·19	0·19	6·8	6·7	0·19	0·19	6·5	6·1
15	0·19	0·19	6·2	6·3	0·19	0·19	6·5	6·6
16	0·19	0·19	6·6	6·2	0·19	0·19	6·4	6·2
17	0·19	0·19	6·7	6·4	0·19	0·19	6·5	6·7
18	0·19	0·19	6·4	6·3	0·19	0·19	6·5	6·8
19	0·19	0·19	6·3	6·1	0·19	0·19	6·3	6·1
20	0·19	0·19	6·2	6·0	0·19	0·19	6·2	6·0
21	0·19	0·19	6·3	6·5	0·19	0·19	6·3	6·0
22	0·19	0·19	6·5	6·4	0·19	0·19	6·5	6·4
23	0·19	0·19	6·5	6·6	0·19	0·19	6·4	6·4
24	0·19	0·19	6·4	6·2	0·19	0·19	6·4	6·5
25	0·19	0·19	6·4	6·1	0·19	0·19	6·1	6·6
26	0·19	0·19	6·1	6·2	0·19	0·19	6·0	6·7
27	0·19	0·19	6·2	6·0	0·19	0·19	6·3	6·2
28	0·19	0·19	6·3	6·1	0·19	0·19	6·2	6·1
29	0·19	0·19	6·2	6·1	0·19	0·19	6·2	6·2
30	0·19	0·19	6·1	6·2	0·19	0·19	6·1	6·1
	Operation no. 3				Operation no. 4			
1	0·19	0·20	12·7	9·1	0·19	0·11	13·7	Nil
2	0·19	0·20	14·0	10·2	0·19	0·11	11·2	Nil
3	0·19	0·20	13·2	11·6	0·19	0·11	9·1	Nil
4	0·19	0·20	12·1	12·2	0·19	0·12	10·4	Nil
5	0·19	0·20	12·9	12·6	0·19	0·12	8·9	Nil
6	0·19	0·19	12·7	12·5	0·19	0·13	10·0	Nil
7	0·19	0·19	13·1	13·0	0·19	0·12	11·7	Nil
8	0·19	0·19	12·8	13·1	0·19	0·13	12·8	Nil
9	0·19	0·19	12·9	13·0	0·19	0·13	12·1	Nil
10	0·19	0·19	13·2	12·9	0·19	0·13	11·6	Nil
11	0·19	0·20	12·7	12·9	0·19	0·13	12·0	Nil
12	0·19	0·19	11·6	12·8	0·19	0·13	12·3	Nil
13	0·19	0·19	14·0	13·9	0·19	0·13	13·0	Nil
14	0·19	0·19	12·7	12·9	0·19	0·14	12·5	Nil
15	0·19	0·19	12·1	12·0	0·19	0·14	12·4	Nil
16	0·19	0·19	12·8	12·6	0·19	0·13	12·6	Nil
17	0·19	0·19	12·6	12·7	0·19	0·14	12·8	Nil
18	0·19	0·19	12·0	11·8	0·19	0·14	13·4	Nil
19	0·19	0·19	14·1	13·5	0·19	0·14	12·0	Nil
20	0·19	0·19	13·2	13·4	0·19	0·15	11·9	Nil
21	0·19	0·19	11·9	12·2	0·19	0·15	11·9	Nil
22	0·19	0·19	12·8	12·6	0·19	0·15	12·8	Nil
23	0·19	0·19	13·7	13·1	0·19	0·15	14·1	Nil
24	0·19	0·19	12·6	12·7	0·19	0·15	12·7	Nil
25	0·19	0·19	12·5	12·6	0·19	0·16	13·3	Nil
26	0·19	0·19	11·2	11·0	0·19	0·15	13·6	Nil
27	0·19	0·19	13·6	13·0	0·19	0·16	12·1	Nil
28	0·19	0·19	12·4	12·5	0·19	0·16	11·9	Nil
29	0·19	0·19	12·0	12·1	0·19	0·16	13·0	Nil
30	0·19	0·19	12·0	12·2	0·19	0·16	12·7	Nil



Text-fig. 3. Following epididymal obstruction the seminiferous tubules become transiently dilated. The magnitude and duration of the dilatation depend on the level of obstruction, becoming greater as the site moves towards the testis. Infarction is followed by immediate tubular shrinkage which is to some extent slowly recovered from.

Unilateral ligation of the cauda epididymidis (operation no. 3) is followed by an immediate weight gain of 15% (Text-fig 2). Thereafter testicular weight remains irregularly elevated, with some tendency to return towards normal, over the 4 weeks covered by the present data.

Infarction of the testis (operation no.4) results in a sudden weight loss of some 58.1% over the first 5 days (Pl. 3, fig. 9). The weight of the operated testis then falls somewhat irregularly to about 20-30% of the weight of the control testis (Text-fig. 2).

The control or sham operation (operation no. 5) is without effect on testicular weight.

(2) Seminiferous tubule diameter

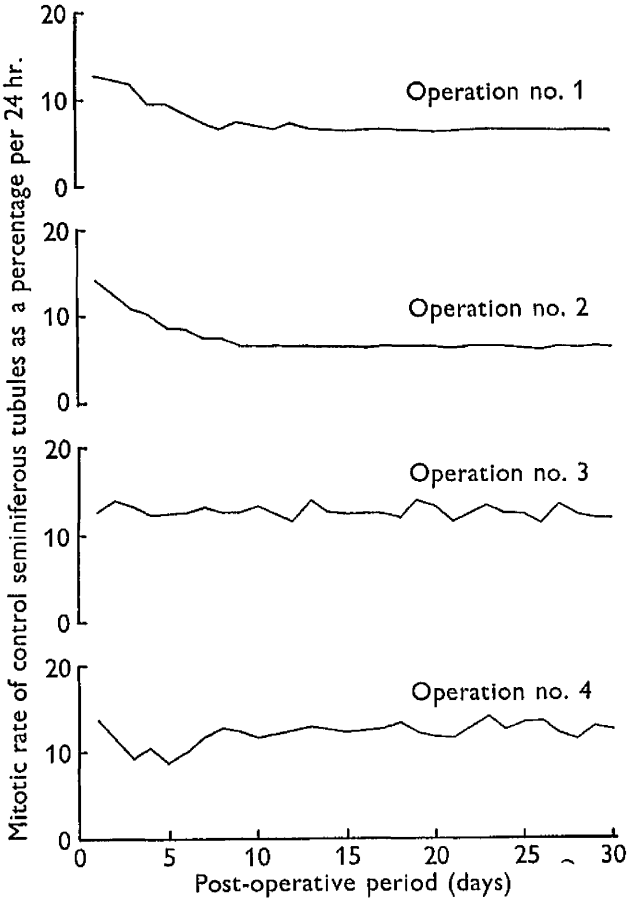
The measured diameters of the seminiferous tubules are presented in Table 2 and Text-fig. 3. After obstruction of the initial segment on one side the average diameter of the seminiferous tubules of the unobstructed opposite testes remains constant at 0.19 mm. On the operated side, seminiferous tubule diameter increases to 0.21 mm. immediately and 0.22 mm. ultimately before returning to the control level of 0.19 mm. on the 13th post-operative day. Similarly, unilateral obstruction just distal to the proximal lobules of the caput is without effect on the average diameter (0.19 mm) of the

seminiferous tubules of the unobstructed testis; those of the obstructed testis, however, increase in diameter from 0.19 to 0.22 mm. Return to the original or control diameter takes place on the 12th post-operative day. Unilateral obstruction of the cauda epididymidis, as before, has no effect on the diameter of the unobstructed contralateral seminiferous tubules but causes those of the operated side to expand slightly to 0.20 mm. This dilatation is transient and subsides by the 6th post-operative day.

Unilateral testicular infarction results in immediate diminution in the diameter of the seminiferous tubules of the affected side: this is reflected by a fall in the seminiferous tubule diameter from 0.19 mm. (control constant) to 0.11 mm. on the 1st post-operative day. From the 4th post-operative day onwards the tubules of the infarcted testis increase gradually in diameter to reach 0.16 mm. 27 days after operation (Text-fig. 3). The control operation (operation no. 5) has no effect on the diameter of the seminiferous tubules of either testis which remains constant at 0.19 mm.

(3) The mitotic pattern

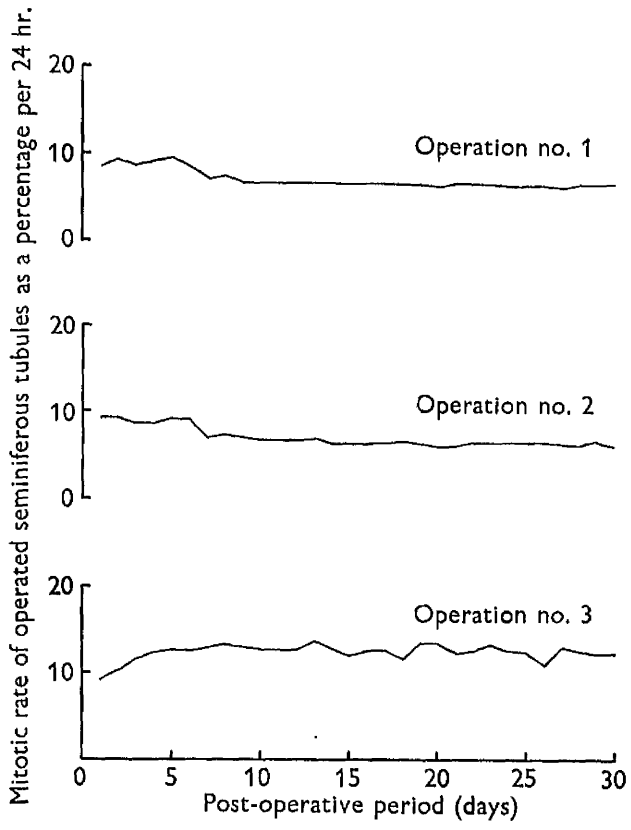
All the mice tolerated the stated dose of colchicine well and the mitotic rate of the basal layer of the



Text-fig. 4. The mitotic rate of the seminiferous epithelium of the control testes shows a progressive reduction following obstruction of the initial segment or at a point just distal to the proximal lobules of the head. Unilateral obstruction of the cauda epididymidis or infarction have no effect on the contralateral seminiferous tubule mitotic rate.

germinal epithelium is summarized as a percentage per 24 hr. in Table 2. After unilateral ligation of the initial segment of the caput the mitotic rate of the seminiferous epithelium of the obstructed testis is about 8 or 9% for the 1st 6 days, falling gradually to 6% about the 14th post-operative day, at which level it remains for the rest of the period surveyed. The corresponding mitotic rate for the contralateral unoperated gonad falls from around 12% initially to a similar low level on the 14th post-operative day (Text-fig. 4). Unilateral obstruction just distal to the proximal lobules results in a comparable mitotic rate for the obstructed testis initially of 9% per day falling to 6% or thereby on the 14th post-operative day and remaining there for the ensuing 2 weeks. The mitotic rate of the seminiferous epithelium of the intact testis falls from 12 or 14% per day initially to reach almost the same mitotic rate as the operated gonad from the 7th day onwards.

Unilateral low epididymal ligation has no effect on the mitotic rate of the germinal epithelium of the intact gonad. On the other hand, that of the obstructed testis exhibits a transient fall in the mitotic rate to begin with, followed by a rise to control levels (9-14% per day) after 4 days.



Text-fig. 5. The mitotic rate of the seminiferous epithelium of the operated gonads shows an immediate reduction after obstruction of the epididymis at all three levels. This is followed by a continuing fall in the case of the two high level obstructions and by a return to normal after obstruction of the cauda.

Infarction naturally terminates mitotic activity in the tubules of the ischaemic testis; the intact gonad returns a basal layer mitotic rate of 9-14% per day. Following the control operation (operation no.5) the seminiferous tubules of both testes exhibit identical mitotic activity, the rate being 9-14% per day. No Leydig mitoses were seen in any of the above series.

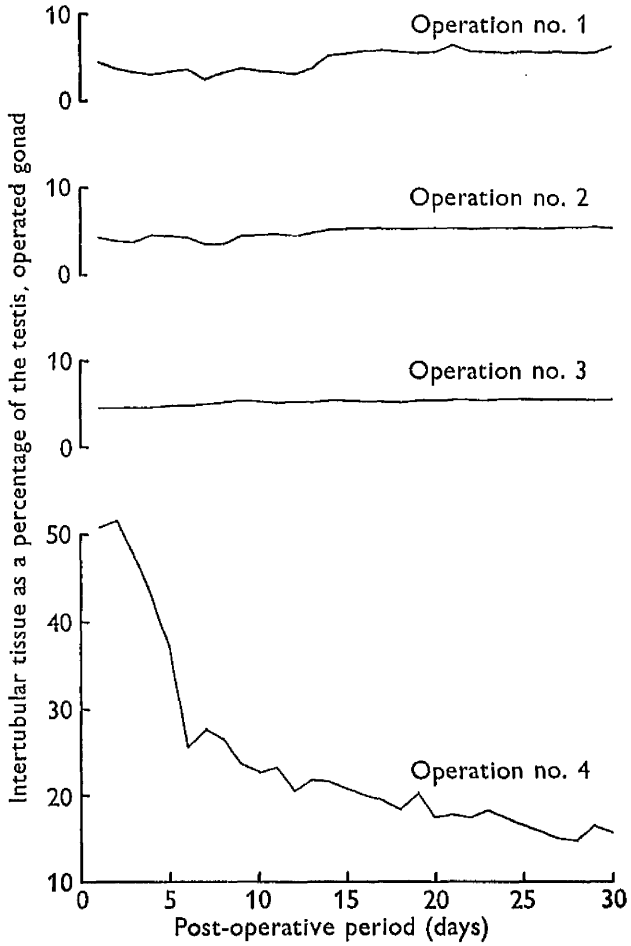
(4) Seminiferous and intertubular tissue masses

After unilateral obstruction of the initial segment (operation no.1), the proportion of the intact control testis accounted for by the seminiferous tubules remains constant at 94.6% and that constituted by the intertubular tissues (blood vessels, connective tissue and Leydig tissue) at 5.4%. On the obstructed side, however, during the period of initial weight gain (Table 3 and Text-fig. 6) the relative amount of seminiferous tubular tissue rises and that of the intertubular tissue falls (e.g. 97.4 and 2.6% respectively). This trend is slightly reversed during the second, or compensated, half of the period studied due to slight tubular atrophy.

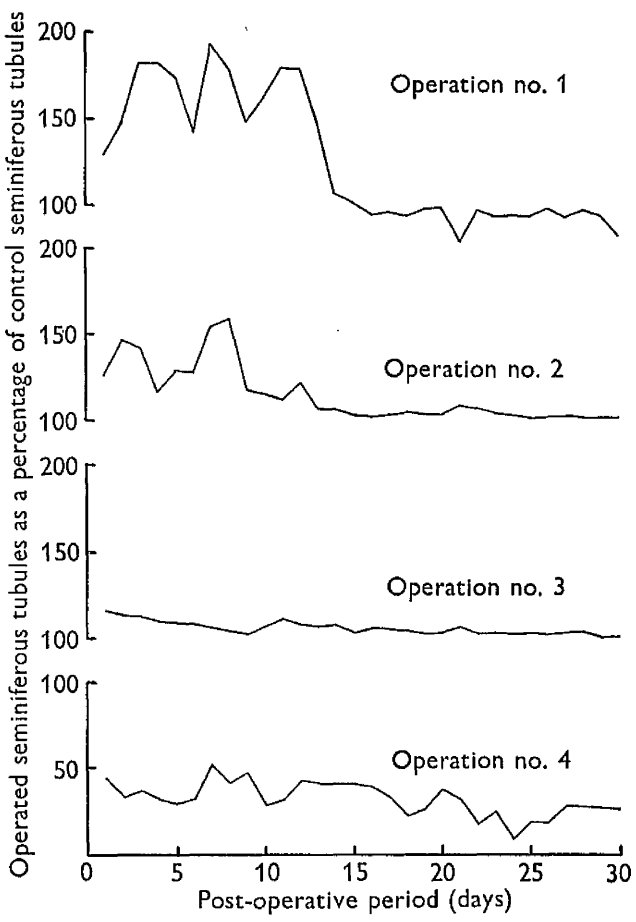
Obstruction just distal to the proximal lobules of the head (operation no. 2) and of the cauda epididymidis (operation no. 3) are attended by similar sequelae (Table 3, Text-figs. 6,7), the initial seminiferous tubule expansion

Table 3. *An analysis of the tubular and intertubular tissues in the post-operative testis*

Post-operative period (days)	Interstitium as a % of operated testis	Weight of interstitium of operated testis (mg.)	Weight of tubules of operated testis (mg.)	Operated tubules as a % of control tubules	Interstitium as a % of operated testis	Weight of interstitium of operated testis (mg.)	Weight of tubules of operated testis (mg.)	Operated tubules as a % of control tubules
	Operation no. 1				Operation no. 2			
1	4·3	10·5	232·5	129·1	4·2	9·0	205	126·1
2	3·8	10·2	258·8	147·1	3·9	11·5	282·5	146·2
3	3·1	9·0	282	182·0	3·8	11·4	289·6	142·4
4	3·0	9·6	310·4	181·3	4·6	10·5	205·5	116·5
5	3·2	10·5	318·5	173·6	4·2	11·1	252·9	128·5
6	3·8	9·6	242·4	140·6	4·1	11·1	259·9	121·2
7	2·6	8·7	327·3	193·2	3·4	9·2	262·8	154·3
8	3·1	11·0	345·0	177·8	3·3	11·2	326·8	158·4
9	3·8	11·4	289·6	147·9	4·5	10·0	212·0	118·0
10	3·3	11·0	324·0	162·4	4·6	10·2	211·8	115·4
11	3·1	11·2	349·8	178·6	4·8	10·3	203·7	112·0
12	3·0	11·6	373·4	177·8	4·1	10·7	251·3	122·9
13	3·7	10·3	267·7	148·1	4·9	10·4	202·6	107·1
14	5·1	11·9	221·1	105·8	5·1	10·8	201·2	105·8
15	5·3	10·4	186·6	100·1	5·2	10·4	190·6	103·2
16	5·8	10·7	174·5	94·5	5·3	11·0	197·0	102·1
17	5·9	12·1	193·9	95·3	5·2	10·8	198·2	104·7
18	5·7	10·5	173·5	93·4	5·2	10·1	183·9	105·0
19	5·5	12·5	214·5	97·7	5·2	10·5	191·5	103·2
20	5·7	12·4	204·6	98·4	5·3	11·4	205·6	103·0
21	6·3	9·5	144·5	77·1	5·2	11·3	207·7	109·1
22	5·7	12·9	213·1	96·3	5·1	9·8	182·2	107·6
23	5·7	10·1	166·9	93·8	5·3	9·6	172·4	104·1
24	5·6	12·9	209·1	94·7	5·3	11·2	200·8	103·5
25	5·7	11·9	197·1	94·3	5·4	11·4	200·6	100·9
26	5·5	9·6	164·4	97·1	5·2	9·9	180·1	102·4
27	5·7	12·4	204·6	92·0	5·3	11·7	209·3	102·9
28	5·6	11·2	171·8	96·6	5·3	10·8	192·2	100·6
29	5·6	9·5	160·5	94·8	5·4	12·0	211·0	101·4
30	6·2	10·3	155·7	82·3	5·3	11·0	196·0	101·6
	Operation no. 3				Operation no. 4			
1	4·6	9·9	205·1	116·0	50·9	85·5	82·5	44·9
2	4·7	12·2	248·8	113·9	51·4	72·9	69·1	33·9
3	4·7	10·3	208·7	113·7	47·9	57·9	63·1	36·4
4	4·8	8·6	170·4	109·8	42·4	46·2	62·8	31·8
5	4·9	10·9	211·1	109·4	36·9	34·3	58·7	27·9
6	4·9	10·6	205·4	107·0	25·5	31·1	65·9	31·7
7	5·0	10·6	200·4	105·9	27·9	36·8	95·2	52·7
8	5·1	10·2	191·8	104·0	26·4	31·9	89·1	40·4
9	5·2	11·1	201·9	102·2	23·8	28·3	90·7	47·9
10	5·1	11·1	225·9	106·9	22·6	17·4	59·6	27·3
11	5·0	10·8	204·2	112·3	23·1	23·1	76·9	32·3
12	5·1	11·8	220·2	108·2	20·3	20·3	79·7	43·4
13	5·1	11·5	214·5	106·4	21·9	24·1	85·9	40·9
14	5·3	11·5	205·5	107·0	21·6	19·6	71·4	40·1
15	5·2	11·7	213·3	103·9	20·9	18·8	71·2	40·6
16	5·3	11·4	222·6	105·5	20·0	17·8	71·2	39·4
17	5·3	10·6	189·4	105·3	19·4	15·3	52·7	33·7
18	5·2	10·4	193·6	104·8	18·3	8·9	40·1	22·3
19	5·3	9·9	177·1	103·4	20·1	12·8	51·2	25·3
20	5·3	11·6	207·4	104·3	17·4	15·5	52·5	37·6
21	5·4	10·6	185·4	107·6	17·9	12·9	59·1	31·6
22	5·3	11·4	203·6	102·4	17·4	6·8	32·2	17·2
23	5·3	9·7	173·3	103·7	18·1	10·5	47·5	25·0
24	5·4	13·6	239·4	102·4	17·3	3·6	17·4	9·6
25	5·3	9·5	170·5	104·2	16·4	6·3	31·7	17·9
26	5·4	11·6	202·4	102·4	15·9	6·7	35·3	17·8
27	5·3	11·5	206·5	104·0	15·0	10·3	48·7	27·7
28	5·4	12·0	213·0	104·7	14·8	7·8	45·2	26·7
29	5·3	11·2	200·8	100·0	16·1	12·9	51·1	26·4
30	5·3	10·6	189·4	100·4	15·9	9·7	51·3	25·4



Text-fig. 6. Epididymal obstruction is followed by no change in the absolute amount of intertubular tissue present; in consequence, when the seminiferous tubules are expanded, the relative amount of intertubular tissue is reduced. This applies to the phase of initial weight gain following all levels of obstruction and to the phase of persistent weight gain after the lower two occlusions. Tubular atrophy following infarction results in an apparent interstitial prominence: this is present to a minimal degree in the compensated phase following obstruction of the initial segment.



Text-fig. 7. The mass of the seminiferous tubules behaves in the same way as testicular weight following the various operations (cf. Text-fig. 2).

becoming less marked as the site of obstruction moves distally. The intertubular tissues in the operated testis are reduced relative to the tubular tissues, as before, during the phase of tubular expansion; they remain, however, in the region of 100% of the control mass.

Testicular infarction is followed in the first 2 days by a sudden reduction in the proportion of the operated testis constituted by the seminiferous tubules (from 94.6 to 48.6%) and by a relative or apparent increase in the proportion of intertubular tissues present. Thereafter, this trend is reversed gradually and the seminiferous tubules comprise 81.4% (control 94.6) of the experimental testis, 30 days after operation. When the seminiferous tubular tissue mass of the operated gonad is expressed as a percentage of that of the control testis, it is seen to undergo a progressive reduction from 49.9% on the 1st post-operative day to 25.4% on the 30th (Text-fig. 7). The intertubular tissues undergo a similar progressive atrophy.

The control operation (no. 5) has no effect on the relative or absolute distribution of tubular and intertubular tissues in the testis.

(5) Histology of operated testes

Ligation of the initial segment (operation no. 1) is followed by a characteristic series of histological changes during the initial 12 post-operative days. For descriptive purposes the seminiferous tubule, in transverse section may be considered to consist, from without inwards, in a PAS positive basement membrane, the germinal epithelium with its six or seven cell layers and the tubule lumen.

The seminiferous tubules are seen to dilate on the 1st post-operative day and they tend to become flattened at points of contact with one another, assuming a somewhat hexagonal contour. The germinal epithelium becomes thinned and is only three or four cells thick by contrast with the control six or seven layers. The individual cellular components of the seminiferous epithelium show no changes of note and all stages of spermiogenesis (Leblond & Clermont, 1952) can be seen. The central lumen of the seminiferous tubules expands considerably; in some tubules it comes to contain large groups or clumps of shed spermatozoa which appear to become detached from the germinal epithelium with their heads bound together with cytoplasm derived from the Sertoli syncytium (Pl. 1, fig. 2). No other cell types are shed. In the lumina of tubules containing shed spermatozoa

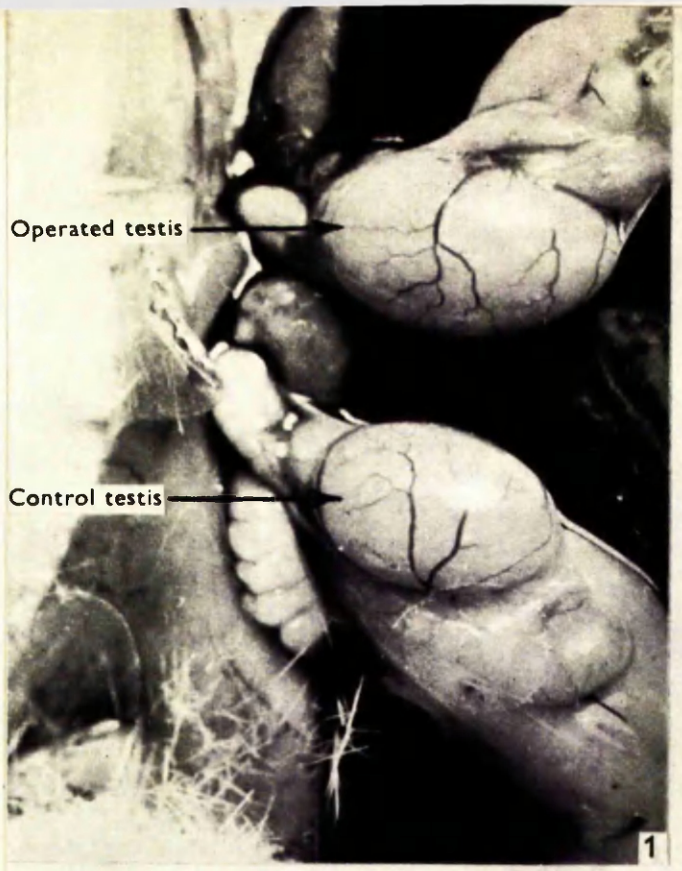


PLATE I

Fig. 1. Whole mount of testes, op. no. 1. Note testicular enlargement 3 days after epididymal obstruction. × 4.

Fig. 2. Operated testis, PAS, op. no. 1. The seminiferous tubules are dilated and contain masses of shed spermatozoa 2 days after obstruction. × 250.

Fig. 3. Operated testis, PAS, op. no. 1. Ten days after obstruction the shed spermatozoa are less evident. × 150.

Fig. 4. Operated testis, PAS, op. no. 1. Twelve days after ligation the testis is fully compensated and tubular architecture is restored. × 150.

peculiar small irregular PAS positive extracellular granules appear. These resist digestion with hyaluronidase and diastase together with methanol chloroform extraction.

The above changes are less evident by the 10th post-operative day (Pl. 1, fig. 3) while by the 12th day the spermatozoa have disappeared from the seminiferous tubule lumen, the lumen is reduced to its former proportions, the germinal epithelium has returned to its previous six or seven cell layer organisation and the sperm whorls are reconstituted (Pl. 1, fig. 4). Histologically, after the 12th day the operated and control testes are indistinguishable.

Removal of free spermatozoa from the lumen of the seminiferous tubules is achieved by a process of phagocytosis. Cells resembling macrophages appear in the seminiferous epithelium on the 3rd or 4th day after the onset of epididymal obstruction. These cells have a finely granular cytoplasm which stains a pale pink with Schiff's reagent and which is devoid of PAS positive granules: the cell membrane is well defined. By the 5th post-operative day these cells are free in the lumen of the seminiferous tubules and they soon become surrounded by clumps of spermatozoa which clearly adhere closely to the macrophage cell membrane (Pl. 2, fig. 5). The spermatozoa so caught appear to be

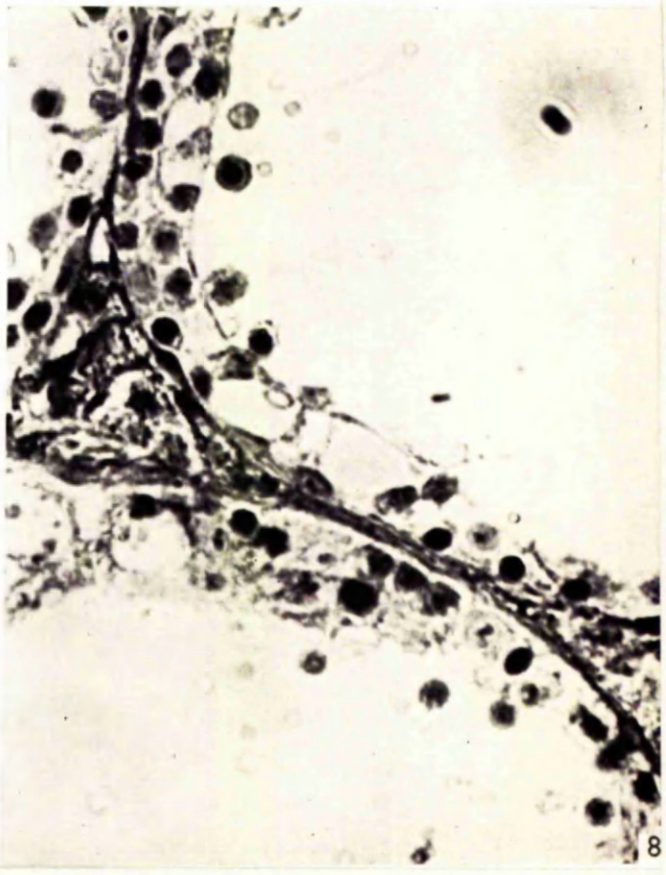


PLATE 2

Fig. 5. Operated testis, PAS, op. no. 1. A macrophage in a seminiferous tubule 6 days after obstruction surrounded by shed spermatozoa. × 600.

Fig. 6. Operated testis, PAS, op. no. 1. A later stage in the phagocytosis of spermatozoa 8 days after obstruction. The macrophage has a kidney-shaped nucleolus and its cytoplasm contains engulfed sperm nuclei. × 600.

Fig. 7. Operated testis, PAS, op. no. 1. As the sperm nuclear material disappears from the macrophage cytoplasm a rosette of PAS positive granules appears. × 600.

Fig. 8. Control testis, PAS, op. no. 2. Occasionally, severely damaged seminiferous tubules occur. × 600.

engulfed head first and in later testes one can see these peculiar cells with residual sperm tails adherent to their cell membranes and cytoplasmic inclusions which stain with haematoxylin clearly distinct from their own nucleus with its membrane, nucleoli and chromatin clumps (Pl. 2, fig. 6). As the tubules are cleared of spermatozoa these cells become progressively larger, their nuclei pronouncedly eccentric and their cytoplasm acquires clumps of PAS positive granules of various sizes (Pl. 2, fig. 7). It is not clear whether these granules are related to the PAS positive granules described above lying free in the lumen of the seminiferous tubules and associated with detached spermatozoa.

Quite exceptionally, an operated or control testis may contain two or three severely damaged tubules lying adjacent to one another (Pl. 2, fig. 8). The seminiferous epithelium in such tubules is one or two cell layers thick and contains peculiar large spaces which are apparently fluid filled. No spermatozoa are visible in such tubules.

The intertubular tissue is less prominent in the operated than in the control testes during the first 12 days after operation. No noteworthy changes were seen in the basement membrane of the seminiferous tubules, the connective tissue ground substance or the blood vessels.

The vast majority of Leydig cells retain their characteristic appearance when subjected to the PAS reaction; the abundant foamy cytoplasm stains a pale pink colour and a few cells contain bright red, diastase resistant, refractile granules. The nuclei have no PAS positive material. In experimental testes some Leydig cells contain large PAS reactive granules dispersed through their cytoplasm. These appear to be connected, by intermediate forms, with abnormally large rounded Leydig cells whose cytoplasm is packed full of PAS reactive granules. These abnormal Leydig cells have from one to five densely staining nuclei and are, in turn apparently linked, by transition forms, with small rounded cells having PAS positive granules cytoplasm and small densely staining nuclei. The PAS positive material which occurs in those abnormal cells resists digestion with diastase and hyaluronidase and solution methanolchloroform. The abnormal Leydig cells are most common during the second post-operative week.

Obstructions distal to the proximal lobules (operation no. 2) and of the cauda epididymidis are followed by changes similar to the above, differing only in degree and becoming less marked and more transient with the more distal ligations. Testicular infarction results in a quite different histological picture.

Within 24 hr. of ligating the testicular artery all the seminiferous tubules become shrunken and the central lumen disappears completely (Pl. 3, fig.10). The rapidity of tubular shrinkage is not the least striking of its features. Cell detail is surprisingly well preserved and it is some days before the phenomena traditionally described as karyolysis, karyorrhexis and pyknosis are clearly established. The basement membrane of the seminiferous tubules become corrugated, irregular in thickness and exhibits a reduced affinity for Schiff's reagent within 2 days of operation. By the 9th post-operative day the seminiferous tubules are obviously less shrunken than those in the immediately post-operative testes and a lumen has reappeared in a small minority. While most nuclear staining is not now discernible, nuclear and cell 'ghosts' are clearly visible (Pl.3, fig.11). The basement membrane is unrecognizable in places. Autolysis and expansion of the seminiferous tubules continues so that, by the 24th post-operative day, they are represented by an amorphous mass which colours faintly with the PAS reaction and has no basement membrane (Pl.3, fig.12).

In the intertubular spaces the blood vessels are greatly dilated within 24 hr. of operation and red blood cells are extensively extravasated throughout the inter-tubular spaces and beneath the tunica albuginea. The

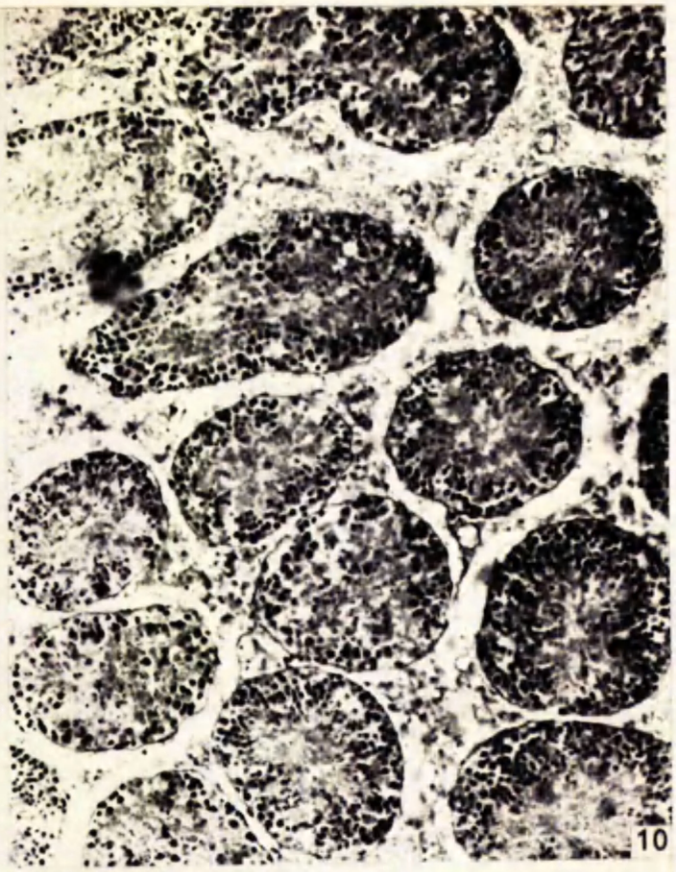


PLATE 3

Fig. 9. Whole mount of testes, op, no. 4. The infarcted gonad loses weight rapidly: 5 days post-operative. × 4.

Fig. 10. Operated testis, PAS, op. no. 4. The seminiferous tubules collapse and lose their lumen 1 day after infarction. × 150.

Fig. 11. Operated testis, PAS, op. no. 4. Note the loss of nuclear staining 9 days after operation. × 150.

Fig. 12. Operated testis, PAS, op. no. 4. Twenty-four days after infarction the tubules are an amorphous mass. × 150.

Leydig cells are shrunken with irregular crenated cytoplasm which stains more intensely than usual with the PAS reaction. Their nuclei disintegrate about the 5th post-operative day and few are recognizable as Leydig cells thereafter. On the 14th day after operation leucocytes appear in the interstitium, and capillaries and mesenchyme follow 10 days later from adhesions between the tunica albuginea and the parietal tunica vaginalis. Connective tissue cells and fibroblasts in the superficial layers of the tunica albuginea appear to survive throughout the whole period covered by the present observations. The changes described are evenly distributed throughout the whole testis affecting central, peripheral, polar and subepididymal regions evenly. The control operation (no. 5) had no effect on testicular histology.

DISCUSSION

(1) Testicular weight.

In the 4 weeks following all the epididymal ligations two distinct phases in testicular response can be recognized (Table 1 and Text-fig. 2). The first is characterized by testicular swelling of abrupt onset immediately after operation, lasting up to 2 weeks. The

extent and duration of the enlargement clearly depend on the level of epididymal obstruction. This is succeeded by the second, or compensated, phase and the testis during this period remains a little above the control weight, in the case of obstructions of the cauda and of the caput distal to the proximal lobules, and a little below in the case of ligation of the initial segment. In the latter instance a stable plateau is reached (Text-fig. 2) and there is no evidence of continuing atrophy. The low weight of the testis on the 30th post-operative day probably merely represents individual variation. Infarction results in progressive testicular atrophy.

Harrison & Macmillan (1954) recorded that obstruction of the initial segment in the rat results in testicular atrophy, the volume of the experimental testes being 58, 68 and 43% of the volume of the control testes on the 7th, 14th and 28th post-operative days, respectively. These figures correspond closely with those following infarction in the present series (Table 1). It is interesting to note that Harrison (1953) found that ligation of the vasa efferentia in the rat was attended by an increase in size of the operated gonad which lasted 6 days. The present restuls, however, are not wholly comparable with those of Harrison & Macmillan owing to species difference. In

addition, the epididymal obstructions described by these writers are consequent on infarction, not ligation as in the present series. Moreover, in mice, owing to the size of the animal, there is greater difficulty of ligation at the exact site. Animals showing any evidence of infection microscopic or macroscopic at sacrifice, have been excluded from the present series.

It has been shown previously (Baillie,1960) that bilateral ligation just distal to the proximal lobules of the caput is not attended by any significant alteration in the volume of the operated testes at 2 weeks to 6 months when these are compared with testes from normal animals of the same age. The initial phase of testicular expansion which follows ligation just distal to the proximal lobules of the caput has plainly been missed in the previous study. This suggests that experimental procedures on the testis are more likely to yield information when one gonad is preserved intact to serve as a control, provided the limitations of this method are remembered. Comparison of the present with the former study suggests, moreover, that weight is a more accurate indicator of testicular response to experimental manipulation than volume since in the present investigation it has been shown that in fact 2 and 4 weeks after operation the experimental testis is slightly but persistently heavier than the control gonad.

Interruption of the blood supply to the cauda epididymidis leads to epididymal tubular necrosis (Macmillan, 1953) and disruption of sperm transport but has no effect on either the cytology or the polar or equatorial diameters of the operated gonad. Since the present increase of 12.9% in testicular weight 3 days after caudal ligation would undoubtedly be detected by Macmillan's comparable volumetric measurement, it seems that the mouse and rat react differently to caudal obstruction. The progressive weight loss following infarction was anticipated and requires no comment.

(2) Seminiferous tubule diameter.

From Table 2 and Text-fig. 3 it will be seen that epididymal obstruction at all levels is followed by temporary dilatation of the seminiferous tubules and the degree and duration of tubular dilatation increase as the site of obstruction moves towards the testis. This phase of seminiferous tubular dilatation of which there appears to be no previous record, corresponds in magnitude and timing with the phase of testicular expansion. The increase in tubular diameter is due in part to the accumulation of shed spermatozoa in the lumen, at least between the 3rd and the 12th day. Its abrupt onset (e.g. from 0.19 to 0.21 mm. within 24 hr.) following operation, however, together with the existence of many fluid filled seminiferous tubules implies that by far the

major portion of the initial testicular weight gain is due to damming back of fluid secreted by the seminiferous tubules. Testicular infarction on the other hand results in the immediate shrinkage of the seminiferous tubules of the affected side, the diameter falling from 0.19 to 0.11 mm. in 24 hr., accompanied by a loss of the seminiferous tubule lumen. These changes can be explained by a failure of secretion of seminiferous tubular fluid as the circulation comes to a standstill.

(3) The mitotic pattern.

From the sham operation (operation no. 5), from adult mouse testes examined elsewhere (Baillie, 1961b) and from the normal mice the 24 hr. mitotic rate of the basal layer of cells in the seminiferous tubules is seen to be symmetrically equal and surprisingly constant, lying between 8 and 14% with few exceptions. Following epididymal obstruction at the three selected levels, the daily mitotic rate on the operated side falls immediately to below 9%. After operations 1 and 2 it continues to fall gradually to around 6%, but the initial drop is restored following obstruction of the cauda epididymidis. The control testes from the three series show no initial fall in tubular mitotic rate and, after the first 2 or 4 days, the control and operated testes behave similarly. It seems, therefore, that the trauma of operative obstruction of the epididymis

results in an immediate depression in the tubular mitotic rate which is reversed if the obstruction is distal enough. With high ligations (operations 1 and 2) producing marked testicular enlargement a gradual bilateral symmetrical reduction in the tubular mitotic rate occurs. Complete infarction of one testis does not disturb contralateral mitotic activity. It is not clear to what extent the above findings are influenced by factors such as cellular dilution, due to primary spermatocytes moving down towards the basement membrane, and the attenuation of basal cells in a lateral direction referred to above: possibly cell migration plays an important part in determining tubular mitotic rate.

(4) Seminiferous and intertubular tissue masses

From Table 3 and Text-figs. 2 and 7 it is plain that obstruction of the excurrent duct system is followed by an increase in the relative and absolute mass of seminiferous tissue present, which accounts entirely for the testicular weight gain. By contrast, the absolute amount of intertubular tissue present undergoes no change with the result that the proportion of the testis formed by intertubular tissue is apparently reduced. This is true of the phase of initial weight gain at all levels of obstruction and also of the compensated phase following the lower two ligation levels. The compensated phase

following obstruction of the initial segment, however, is characterized by a degree of actual tubule atrophy with a consequent slight apparent increase in the intertubular tissue. There is no actual hypertrophy of the intertubular elements however. Following testicular infarction, on the other hand, the intertubular tissues undergo a measure of actual atrophy: notwithstanding this they represent a much higher fraction of the operated gonad (see Text-fig. 6) than previously.

The results reported in this series after epididymal obstruction agree with those recorded by Slotopolsky & Schinz (1925) who failed to demonstrate hyperplasia and hypertrophy of the interstitial cells following unilateral interruption of the vasa efferentia. In contrast, the findings of Steinach (1929) who reported luxuriant hyperplasia and hypertrophy of the interstitial cells following bilateral obstruction of the vasa efferentia in the senile rat are not confirmed by the present results. The most recent work in this field, however, is equivocal in that Harrison & Macmillan (1954) report a decrease in the total volumeof Leydig cells present following high epididymal obstruction in seven of the nine animals studied and an increase in the remaining two at 28 and 189 days after operation. The present experiments do

not permit of comparison beyond the 30th post-operative day.

The combination of testicular atrophy and apparent interstitial increase following ischaemia described above resembles those changes noted by Bouin & Ancel (1903) after occlusion of the vas deferens and those noted by van Wagenen (1924,1925) and Oslund (1926) after obstruction of the vasa efferentia in the rat. On this basis the criticisms of Moore & Quick (1924-25), who doubted whether factors such as temperature, operative trauma and damage to blood vessels and nerves were excluded from van Wagenen's (1924,1925) experiments, have added point and the author agrees with White's (1933) opinion that occlusion of the excurrent duct system is not solely responsible for the subsequent degenerations noted in the testis.

(5) Histology of operated testes

From the microscopical observations it is plain that much of the initial dilatation of the seminiferous tubules after epididymal obstruction is due to retention of a fluid in the lumen which normally is necessary for the transport of spermatozoa. This dilatation is also in part due to the accumulation of spermatozoa in the lumen. By contrast in the rat following ligation of the vasa efferentia (Harrison, 1953) primary and secondary

spermatocytes are shed in addition to spermatids and spermatozoa, a more severe degree of disruption of the seminiferous epithelium than occurs in the mouse testes. The remarkable process of testicular adaptation, which is fully effective within 14 days of the operation includes a reduced mitotic rate in the seminiferous epithelium in the case of the higher obstructions, reduced secretion into or perhaps increased absorption of fluid from the seminiferous tubules and the phagocytosis of accumulated spermatozoa in the lumina of obstructed seminiferous tubules. It is not clear whether these macrophages are derived from altered Sertoli cells or from the intertubular mesenchyme. It is interesting to note that Oettlé & Harrison (1952) described somewhat similar macrophage-like cells in the tubules of ischaemic rat testes: these cells also contained debris and the yellowish pigment which possibly corresponds with the PAS positive inclusions seen in the present series of macrophages (Pl. 2,fig.7). The PAS positive granules seen in the macrophages of the mouse seminiferous tubules are of a glycoprotein nature and are presumably in some way related to the phagocytic activities of these cells.

The occurrence of occasional severely damaged seminiferous tubules, which resemble those described in the testes of cold stressed mice (Baillie, 1961a) as an

incidental finding, suggests that the abnormal tubules represent some form of spontaneous degeneration peculiar to this strain of mouse. A similar degenerative change in immature mice of this colony is described elsewhere (Baillie, 1961b). After epididymal obstruction no histological changes were seen in the interstitial tissue and no mitoses were observed in the Leydig cells. Abnormal Leydig cells with abundant PAS positive cytoplasmic material, probably glycoprotein in nature, have been discussed fully previously (Baillie, 1960). Their appearance here indicates that they are a constant feature of the early stages of epididymal obstruction. They do not exhibit mitotic activity.

SUMMARY

1. Groups of thirty adult male albino mice were subjected to five unilateral surgical procedures including ligation of the initial segment of the caput epididymidis at the level of the proximal lobules, ligation of the caput at a point immediately distal to the proximal lobules of the head, ligation of the cauda epididymidis, ligation of the testicular artery and transient exteriorization of the testis. One animal from each group was killed daily forming a post-operative series covering 1 month and each received colchicine on the day of sacrifice.

2. After all epididymal ligations two phases in testicular response can be discerned. First, the testis swells and the extent and duration of testicular swelling depend on the level of epididymal obstruction. A compensated phase follows when testicular weight is nearly normal. Infarction results in progressive weight loss.

3. After epididymal ligation the seminiferous tubules undergo a temporary dilatation whose degree and duration is determined by the level of obstruction. The dilatation is due initially to accumulation of fluid, later to retention of spermatozoa. Infarction results in collapse of the seminiferous tubules due to failure of fluid secretion.

4. Normally both testes have the same seminiferous tubular mitotic rate. The trauma of epididymal ligation is thought to produce an immediate transient depression in the mitotic rate of the operated testis. High ligations producing marked testicular enlargement cause a gradual reduction in the mitotic rate of both the operated and control testis. Infarction has no effect on the contralateral mitotic activity.

5. Epididymal obstruction is followed by no change in the absolute amount of intertubular tissue present. Thus, when tubules are dilated the relative amount of intertubular tissue is reduced. This applies to the phase of initial weight gain after obstruction and to the phase of

persistent weight gain after the lower two occlusions. Tubular atrophy following infarction results in an apparent interstitial hypertrophy.

6. Testicular adaptation to epididymal obstruction is fully effective within 14 days and includes a reduced mitotic rate in the seminiferous epithelium, a reduced secretion of fluid into the tubules and the phagocytosis of shed spermatozoa, by macrophages which acquire rosettes of PAS positive cytoplasmic granules. The appearance of atypical Leydig cells with masses of PAS positive material (? glycoprotein) in their cytoplasm is a constant accompaniment of epididymal obstruction.

REFERENCES

BAILLIE, A.H. (1959). The interstitial cell of Leydig. Endeavour Essay. British Association.

BAILLIE, A.H. (1960). The biology of the Leydig cell; histochemical and histological changes following high epididymal obstruction. J. Endocrin. 20, 339-344.

BAILLIE, A.H. (1961a). Histochemical studies of the mouse testis following cold exposure. Scot. med. J. 6, 6-11.

BAILLIE, A.H. (1961b). Observations on the growth and histochemistry of the Leydig tissue in the postnatal prepubertal mouse testis. J. Anat., Lond., 95, 357-370.

BAILLIE, A.H. (1962). Histochemical differentiation of the basement membrane of the mouse seminiferous tubule. Quart. J. micr. Sci. (in the Press).

BOUIN, P. & ANCEL, P. (1903). Recherches sur les cellules interstitielle du testicle des Mammiferes. Arch. zool. exp. gén. 1, 437-526.

BULLOUGH, W.S. (1950). Epidermal mitotic activity in the adult female mouse. J. Endocrin. 6, 340-349.

EBLING, F.J. (1954). Changes in the sebaceous glands and epidermis during the oestrous cycle of the albino rat. J. Endocrin. 10, 147-154.

HARRISON, R.G. (1953). The effect of ligation of the vasa efferentia on the rat testis. Proc. Soc. Stud. Fertil. 5, 97-100.

HARRISON, R.G. & MACMILLAN, E.W. (1954). The effects of high epididymal obstruction upon the Leydig cell volume of the rat testis. J. Endocrin. 11, 89-96.

LEBLOND, C.P. & CLERMONT, Y. (1952). Spermiogenesis of rat, mouse, hamster and guinea pig as revealed by 'periodic acid-fuchsin sulphurous acid' technique. Amer. J. Anat. 90, 167-215.

MACMILLAN, E.W. (1953). The effects of interruption on the vasal and inferior epididymal arteries on the cauda epididymis and testis. Proc. Soc. Stud. Fertil. 5,12-19.

MOORE, C.R. & QUICK, W.J. (1924-25). Properties of the gonads as controllers of somatic and physical characteristics. VII. Vasectomy in the rabbit. Amer.J. Anat. 34, 317-336.

OETTLE, A.G. & HARRISON, R.G. (1952). The histological changes produced in the rat testis by temporary and permanent occlusion of the testicular artery. J. Path. Bact. 64, 273-297.

OSLUND, R.M. (1926). Cryptorchid testes and testicular hormone production. Amer. J. Physiol. 77, 76-82.

SLOTOPOLSKY, B. & SCHINZ, H.R. (1925). Histologisches Zur Steinachunterbindung. A. mikr.-anat. Forsch. 2, 225-253.

STEINACH, E. (1920). Verjüngung durch experimentelle Neubelebung der alternden Pubertätsdrüse. Arch. Entw. Mech. Org. 46, 553-618.

WAGENEN, G. van (1924). Degeneration of germinal epithelium in the testis of the rat as a result of efferent duct ligation. Anat. Rec. 27, 189-190.

WAGENEN, G. van (1925). Changes in the testis of the rat following ligation of the ductuli efferenti. Anat. Rec. 29, 399.

WHITE, E.W. (1933). Duration of fertility and histological changes in reproductive organs after ligation of vasa efferentia in the rat. Proc. Roy. Soc. B, 113, 544-550.

THE HISTOCHEMISTRY AND ULTRASTRUCTURE OF THE GONOCYTE.

by

A.H. BAILLIE.

In the vertebrate male, shortly after the sex c. from the thickened genital epithelium invade the underlying mesenchyme of the genital ridge, andbbefore the cords lose their connection with the surface epithelium, a number of large atypical cells appear in the substance of the cords (Gillman, 1948). These cells were described by Regaud (1901) and Schoenfeldt (1902) and have pale staining spherical nuclei about 9 μ in diameter with globular nucleoli and dust-like chromatin particles, and their cytoplasm is slightly eosinophilic. They were initially termed the primitive sex cells, but the term gonocyte is more widely used currently (Clermont & Perey, 1957). Origins suggested for the gonocyte include the genital epithelium (Felix, 1912), intestinal epithelium (Fuss, 1912), gut endoderm (Willier, 1939), and some writers claim to have traced them as far back in ontogeny as the early cleavage divisions (Bounoure, 1934). They persist in the testis from the formation of the sex cords until a few days after birth when they disappear. Some workers hold that they disintegrate entirely without leaving descendants in the seminiferous tubules (Goldsmith, 1928; Bookhout, 1937; Allen, 1949; Bryson, 1944) while others think that the gonocytes eventually give rise to spermatozoa

(Swift, 1916; Simkin, 1923; Clermont & Perey, 1957). Notwithstanding the undertainty surrounding its origin and fate, the gonocyte undoubtedly is a prominent feature of immature seminiferous tubules, and little is known of its cytochemistry and nothing of its ultrastructure. This paper attempts to describe its histochemical and ultrastructural characteristics.

MATERIAL AND METHODS.

Six male Swiss white mice were killed daily between birth and the end of the second week of postnatal life, a total of ninety animals. The testes were fixed in formol corrosive solution, dehydrated in cellosolve, and impregnated in ester wax. Sections at 5μ were stained with haematoxylin and eosin and the PAS reaction. These sections established that the gonocytes were peculiar to the first eight days of postnatal life in the mouse.

Nine animals aged from birth to eight days were used at daily intervals in the colchicine study. Each animal received an intraperitoneal injection of colchicine (0.1 mg. in water/100 g. body weight) at 10.00 cm. on the appropriate day. They were killed 5 hr. later and their testes examined using the PAS technique. The period chosen was identical with that used by Bullough (1950) and Ebling (1954) who selected it to vitiate the effects of the

diurnal mitotic cycle.

The testes of ten neonatal and ten 7 day old animals were subjected to each of the following histochemical tests: ascorbic acid (Bacchus, 1950); glucose (Muller, 1955); aryl sulphatase (Rutenberg, Cohen & Seligman, 1952); succinic dehydrogenase (Nachlas, Tsou, de Souza, Cheng, and Seligman, 1957); cytochrome oxidase (Moog, 1943); Nachlas, Crawford, Goldstein & Seligman, 1958); 3 β-hydroxysteroid dehydrogenase (Wattenberg, 1958; Baillie & Griffiths, in Press) and esterase using the substrates indoxyl acetate (Holt & Withers, 1952); α-naphthyl acetate (Pearse, 1960); Naphthol AS acetate, Naphthol AS-LC acetate, Naphthol AS-D acetate (Baillie, in Press). In each instance the gonad was "snap frozen" in solid carbondioxide at -20°C, the tissues sectioned at 20 on a cryostat at -20°C, and the sections attached to glass slides by momentary thawing.

In preparation of the ultrastructure age series four animals were used at zero and seven days. Pieces of testis, about 1 mm. cube in size, were fixed in buffered osmic acid (Zetterqvist, 1956), dehydrated in methanol and embedded in araldite (Luft, 1961), and sectioned on Porter-Blum and Huxley microtomes. After staining with

1% methanolic lead acetate the sections were screened on unocated copper brids using a Philips electron microscope EM.75B.

RESULTS.

(1) Haematoxylin and eosin. The gonocytes are present in the neonatal testis seminiferous tubules and remain recognisable up to the eight postnatal day. They are usually to be found in the centre of the primitive tubule surrounded by a palisade of supporting cells (Pl. 1, fig. 1). They are large oval or rounded cells with a well demarcated cell membrane, clear almost glassy cytoplasm which is eosinophilic, and a large nucleus. The nucleus is pale staining with fine dust-like chromatin occasionally arranged in coarser clumps. The nuclear membrane is usually clearly defined. Degenerate gonocytes with damaged and disintegrating or even pycnotic nuclei and more eosinophilic cytoplasm bounded by an irregular and incomplete cell membrane are very occasionally seen, but these represent only a small fraction of the total number of gonocytes.

(2) PAS reaction. This technique adds little to the H.& E. picture. The gonocytes have pale gray-pink cytoplasm with no PAS positive inclusions of any kind.

(3) Results of colchicine administration. Mitotic figures were seen in many gonocytes throughout the period they are present. The mitotic plates appear normal and only exceptional metaphase plates were obviously abnormal. The daily mitotic average was fairly constant between birth and their disappearance at the eighth day and was about 11 per cent. An example of a mitotic gonocyte is to be seen in Plate 1, fig. 2.

(4) Histochemistry. Tiny particles of silver denoting the presence of ascorbic acid are deposited in the cytoplasm of some of the gonocytes. The majority, however, contain no ascorbic acid. Glucose and aryl sulphatase are not present in histochemically demonstrable amounts. Using the substrates dehydro-epiandrosterone pregnenolone and 17α-hydroxy pregnenolone, no 3β-hydroxy-steroid dehydrogenase was found. Esterase activity was not demonstrates with any of the substrates used.

(5) Ultrastructure. When the seminiferous tubules are examined in the neonatal period with the electron microscope, the gonocytes are the most striking cells encountered. They are large rounded cells, usually central in position, and they have at least one and frequently two large atypical and quite characteristic nucleoli (Pl. 1, Fig. 3).

The nuclear membrane (Pl. 2, fig. 4) consists clearly of two discrete perforate layers separated by material of lesser electron absorption. The pores in the two layers are usually coincident with one another. The homogeneous nuclear contents present no features of note: occasionally one is fortunate enough to find organisation of nuclear contents into chromosomes in the early stages of mitosis. The nucleoli are large rounded structures with no obvious limiting membrane (Pl. 2, fig. 4). They have the appearance of a sponge, and the pores contain apparently typical nuclear material which is of much lesser electron absorption than the nucleolar material itself. The nucleolar material is very electron dense, and to a cursory inspection appears faintly granular. Closer inspection of the nucleolar material adjacent to and overlapping the pores, however, reveals that the granules are in reality rather elongated electron dense bodies with material of lesser electron density in the interior. No connections between the nucleoli and the nuclear membrane were seen, and no evidence of extrusion of the nucleolar material into the cytoplasm was found.

The ergastoplasm is unremarkable: occasional membranes, perhaps derived from the endoplasmic reticulum are seen

(Pl. 2, fig. 5). The Golgi apparatus is not a prominent feature of these cells: small portions of Golgi membranes are seen from time to time, but vacuoles and vesicles in relation to these membranes are conspicuously absent. The gonocyte mitochondria (Pl. 3, fig. 6) are rounded or, more usually, slightly elongated with a double limiting membrane and lamellar cristae. Near the plasma membrane small groups of membrane limited vesicles are sometimes found (Pl. 3, fig. 6).

DISCUSSION.

On the basis of the present material it is not possible to offer comment on the origin of the gonocyte. The cytology of the cell as revealed by haematoxylin and eosin is well known (Gillman, 1948) and requires no elaboration. The scarcity of degenerating gonocytes in the mouse seminiferous tubules contrasts with Clermont and Perey's (1957) experience in the rat when, at any age, as many as two-thirds of the gonocytes were in the process of disintegration, and is completely at variance with the views of Popoff (1909), Allen (1904), Hargitt (1926), and Kirkham (1915) who maintain that all the gonocytes disintegrate and disappear within a few days of birth. In the mouse they all undoubtedly disappear within eight

days of birth, but the use of colchicine suggests that the disappearance is effected by cell division, rather than disintegration. At this time type A spermatogonia are appearing in the tubules and little mitotic activity is evident in the supporting cells. From a similar situation in the rat, Clermont and Perey (1957) drew the obvious inference that the gonocytes gave rise to type A spermatogonia.

The histochemical features of the gonocyte are of a rather negative nature. With the exception of ascorbic acid, none of the enzymes or constituents sought was present in histochemically demonstrable amounts. The failure to demonstrate succinic dehydrogenase and cytochrome oxidase in this cell when mitochondria are undoubtedly shown to be plentiful in the electron micrographs and by earlier workers using older techniques (Rubaschkin, 1912; Taschaschin, 1910) underlines the shortcomings of these particular histochemical techniques.

Identification of cell types, particularly in immature organs, demands the definition of acceptable and precise criteria. Many such criteria have been suggested for the recognition of gonocytes, including mitochondrial arrangement, the disposition of the Golgi complex, nuclear structure, and the nature of the attraction sphere (Gilman,

1948). Of these various characteristics, only that of size appears to have a universal application. The histochemical features of the gonocyte described above do not materially aid its identification, but the nucleolar structure disclosed by the electron microscope appears to be peculiar and highly specific, at least in the mouse. It remains to be seen, however, whether this organelle has a similar structure in other species.

The structure of the nuclear membrane is common to many other cell types (Callan, 1954) and the nuclear contents likewise. The readiness with which chromosomes can be visualised in these peculiar cells has clear applications in this field of study. The significance of the groups of small vesicles sometimes encountered just inside the plasma membrane of the gonocyte is unclear.

SUMMARY.

The histochemistry and ultrastructure of the gonocyte have been studied in the seminiferous tubules of the mouse during the first eight days of postnatal life. The gonocyte has ascorbic acid in its cytoplasm but no demonstrable glucose, cytochrome oxidase, succinic dehydrogenase, aryl sulphatase, 3β-hydroxysteroid dehydrogenases or esterases. The gonocytes are shown to

undergo mitosis and are thought to give rise to type A spermatogonia. Peculiar nucleolar structure seems to be a characteristic of the gonocyte and is described in detail.

The author is grateful to the Anatomy Department of Glasgow University for the research facilities provided.

REFERENCES.

ALLEN, B.M. (1904) The embryonic development of the ovary and testis of the mammals. Amer. J. Anat., 3, 89-153.

ALLEN, E. (1949) Studies on degenerating sex cells in immature mammals. 1. An analysis of degeneration in primordial and large germ cells in male albino rats aged 1-9 days. J. Morph., 85, 405-422.

BACCHUS, H. (1950) Cytochemical study of the adrenal cortex of the rat under salt stresses. Amer. J. Physiol., 163, 326-331.

BAILLIE, A.H. (In Press). Esterase substrate specificity in the testis of the growing mouse. Quart. J. micr. Sci.

BAILLIE, A.H. & GRIFFITHS, K. (In Press). Evidence for the existence of more than one 3 β-hydroxysteroid dehydrogenase. J. Endocrin.

BOOKHOUT, C.G. (1937) The germ cell cycle in the guinea pig. II. The postnatal development of the testis. Z. Zellforsch., 25, 749-763.

BOUNOURE, M.L. (1934) Recherches sur la lignee germinale chez la grenouille rousse aux premiers stades du developpement. Ann. des sci. nat., zool., 17, 67-245.

BRYSON, V. (1944). Spermatogenesis and fertility in Mus musculus as affected by factors at the T locus. J. Morph. 74, 131-187.

BULLOUGH, W.S. (1950). Epidermal mitotic activity in the adult female mouse. J. Endocrin., 6, 340-349.

CALLAN, H.G. (1954) "Recent work on the structure of cell nuclei" in Fine Structure of Cells, 89-110. Noordhoff, Holland.

CLERMONT, Y., & PEREY, B. (1957) Quantitative study of the cell population of the seminiferous tubules in immature rats. Amer. J. Anat., 100, 241-260.

EBLING, F.J. (1954) Changes in the sebaceous glands and epidermis during the oestrous cycle of the albino rat. J. Endocrin., 10, 147-154.

FELIX, W. (1912) The development of the urogenital organs. In Manual of Embryology, 2, 752-979. Kiebel & Mall, Philadelphia.

FUSS, A. (1912) Ueber die Geschlechtszellen des Menschen und Saugetiere. Arch. f. mikr. Anat., 81, 1-23.

GILLMAN, J. (1948) The development of the gonads in man, with a consideration of the role of foetal endocrines and the histogenesis of ovarian tumours. Contr. Embryol. Carneg. Instn., 210, 81-131.

GOLDSMITH, J.B. (1928) The history of the germ cells in the domestic fowl. J. Morph., 46, 275-315.

HARGITT, G.T. (1926) The formation of the sex glands and germ cells of mammals. II. The history of the male germ cells in the albino rat. J. Morph., 42, 253-306.

HOLT, S.J. & WITHERS, R.J.F. (1952) Cytochemical localisation of esterases using indoxyl derivatives. Nature, Lond., 170, 1012-1014.

KIRKHAM, W.B. (1915) The germ cell cycle in the mouse. Anat. Rec., 10, 217-219.

LUFT, J.H. (1961) Improvement in epoxy resin embedding methods. J. biophys. biochem. Cytol., 9, 409-414.

MOOG, F. (1943) Cytochrome oxidase in early chick embryos. J. cell. comp Physiol., 22, 223-231.

MULLER, G. (1955) Uber eine Vereinfachung der reaktion nach Hale. Acta Histochem., 2, 68-70.

NACHLAS, M.M., CRAWFORD, D.T., GOLDSTEIN, T.P., & SELIGMAN, A.M. (1958) The histochemical demonstration of cytochrome oxidase with a new reagent for the Nadi reaction. J. Histochem. Cytochem., 6, 445-456.

NACHLAS, M.H., TSOU, K.C., de SOUZA, E., & SELIGMAN, A.M. (1957) Cytochemical demonstration of Succinic Dehydrogenase by the use of a new p-nitrophenol substituted ditetrazole. J. Histochem. Cytochem., 5, 420-436.

PEARSE, A.G. (1960) Histochemistry, theoretical and applied. Churchill, London.

POPOFF, N. (1909) L'ovule mâle et le tissu interstitiel du testicle chez les animaux et chez l'homme. Arch. de Biol., 24, 433-500

REGAUD, C. (1901) Etude sur la structure des tubes seminiferes et sur la spermatogenese chez les mammiferes. Arch. d' Anat. micr., 4, 101-156.

RUBASCHKIN, W. (1912) Zur dehre von der Keimbahn bei Säugertieren Über die Entwicklung der Keindrussen. Anat. Hefte, 46, 343-408.

RUTENBERG, A.M., COHEN, R.B. & SELIGMAN, A.M. (1952) Histochemical demonstration of aryl sulfatase. Science, 116, 539-543.

SCHOENFELDT, H. (1902) da Spermatogenèse chez le laureau et chez les mammiferes en general. Arc. Biol., 18, 1-72.

SIMKINS, C.S. (1923) Origin and migration of the so-called primordial germ cells in the mouse and rat. Acta. Zool., 4, 241-278.

SWIFT, C.H. (1916) Origin of the sex cords and definitive spermatogonia in the male chick. Amer. J. Anat., 20, 375-410.

TSCHASCHIN, S. (1910) Ueber die Chondriosomen der Urgeschlechtozellen bei Vögelembryonen. Anat. Anz., 37, 597-621.

WILLIER, B.H. (1939) Embryonic development of sex. In Sex and Internal Secretions (E. Allan, Ed.) 64-144. Baltimore.

ZETTERQVIST, H. (1956) The Ultrastructural Organisation of Columnar Absorbing Cells of the Mouse Jejunum. Karolinska Institutet, Stockholm.

EXPLANATION OF PLATES.

PLATE 1.

Fig. 1. Testis, 2 days, H.& E. A typical gonocyte with abundant clear cytoplasm, and a large vesicular nucleus is seen. X 350.

Fig. 2. Testis, 3 days, P.A.S., after colchicine administration. A typical gonocyte in the centre of the seminiferous tubule is seen in mitosis. X 350.

Fig. 3. Testis, 0 days. The gonocyte (G) is typically large and rounded, centrally placed in the tubule and with two nucleoli. X 4,200.

PLATE 2.

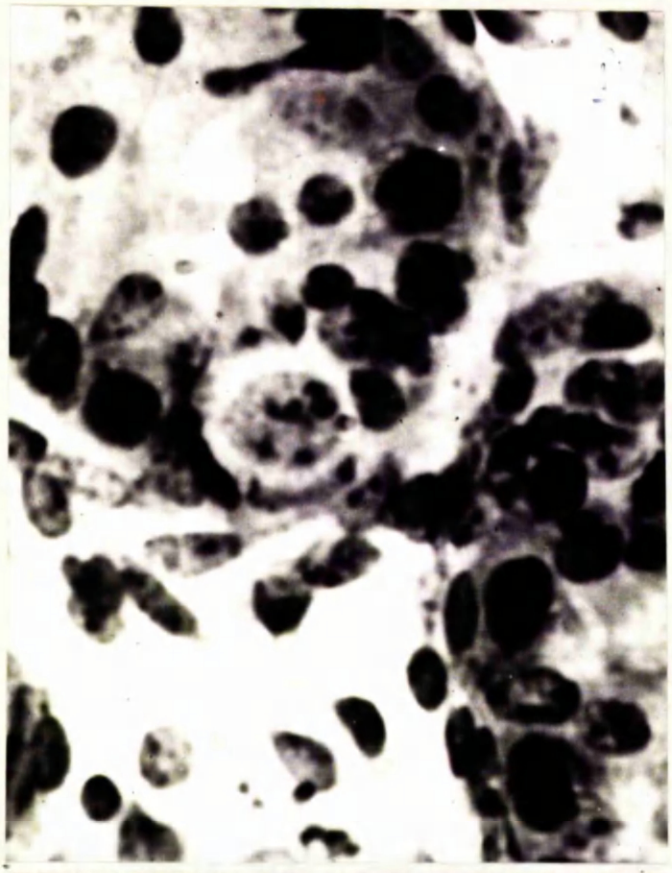
Fig. 4. Testis, 0. days. The double nuclear membrane (M) is a double-layered structure with an intermediate zone of lesser electron density. The "porous" nature of the nucleolus (N) is seen. X 43,000.

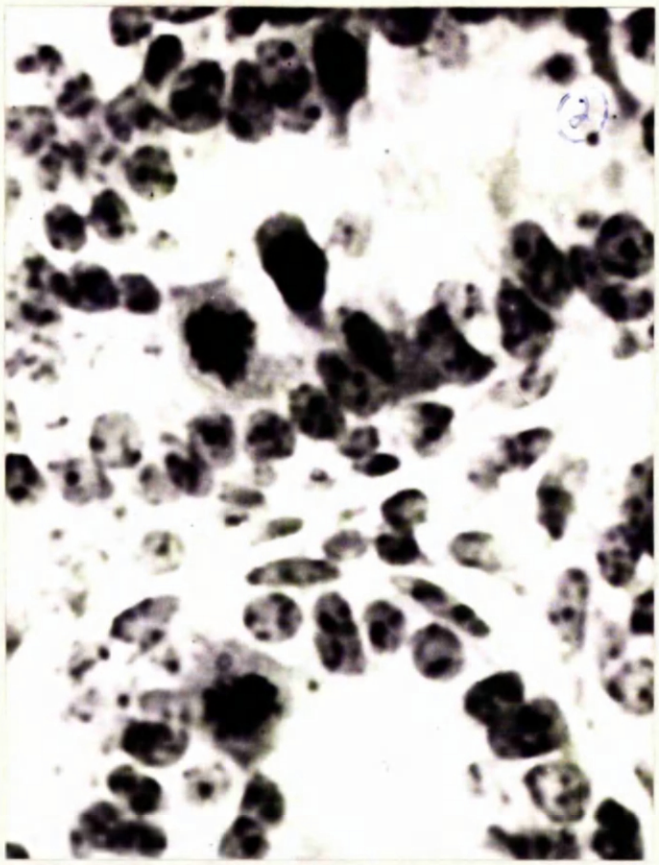
Fig. 5. Testis, 0. days. The ergastoplasm presents no features of note. A membrane (E), perhaps of endoplasmic reticular nature, is seen. X 35,000.

PLATE 3.

Fig. 6. The bilaminate plasma membrane presumably conforms to the Danielli model. The mitochondria (C) are bounded by a double membrane and the interior contains plate-like cristae. A number of small vesicles (V) are seen near the cell membrane. X 43,000.

Plate I.





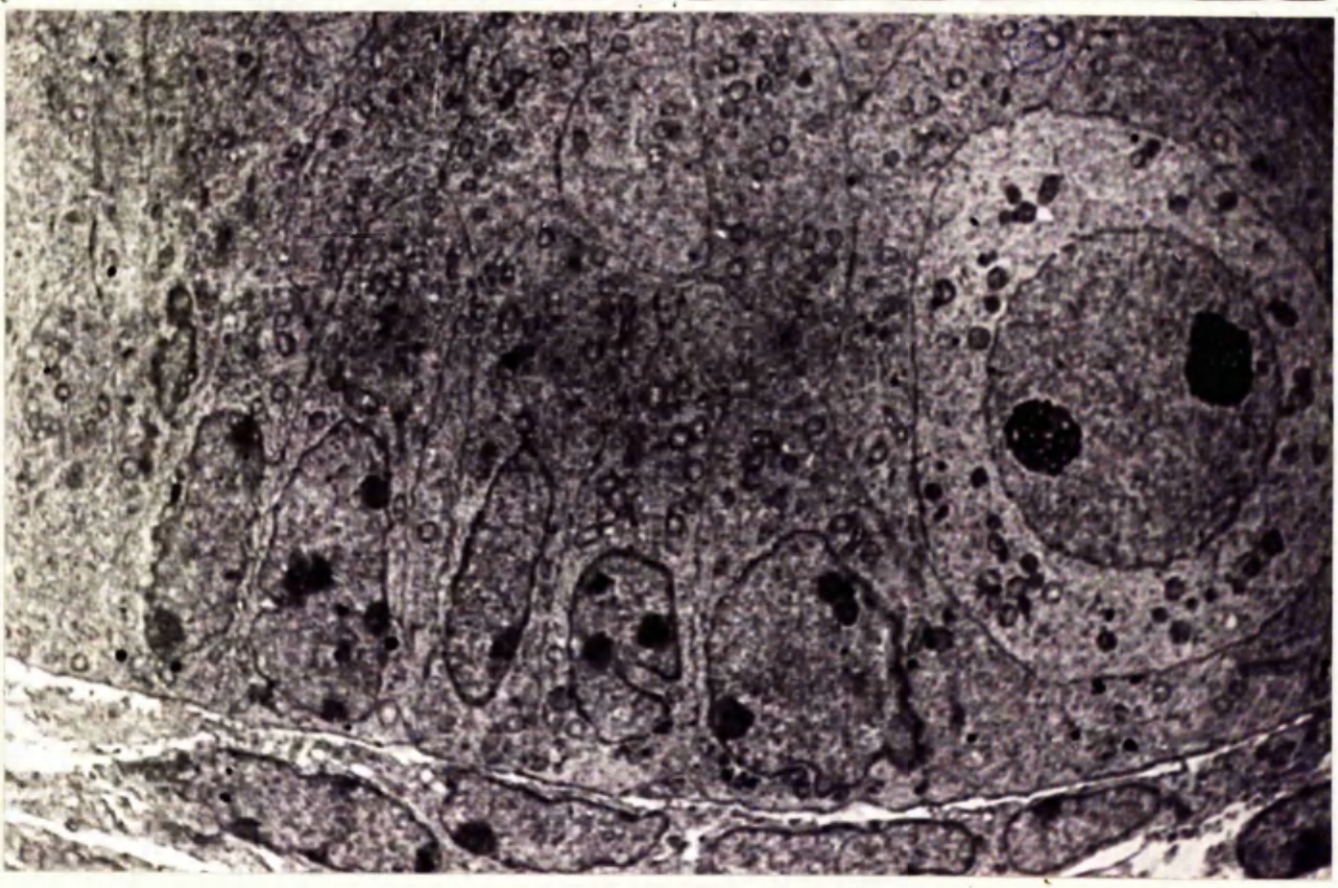
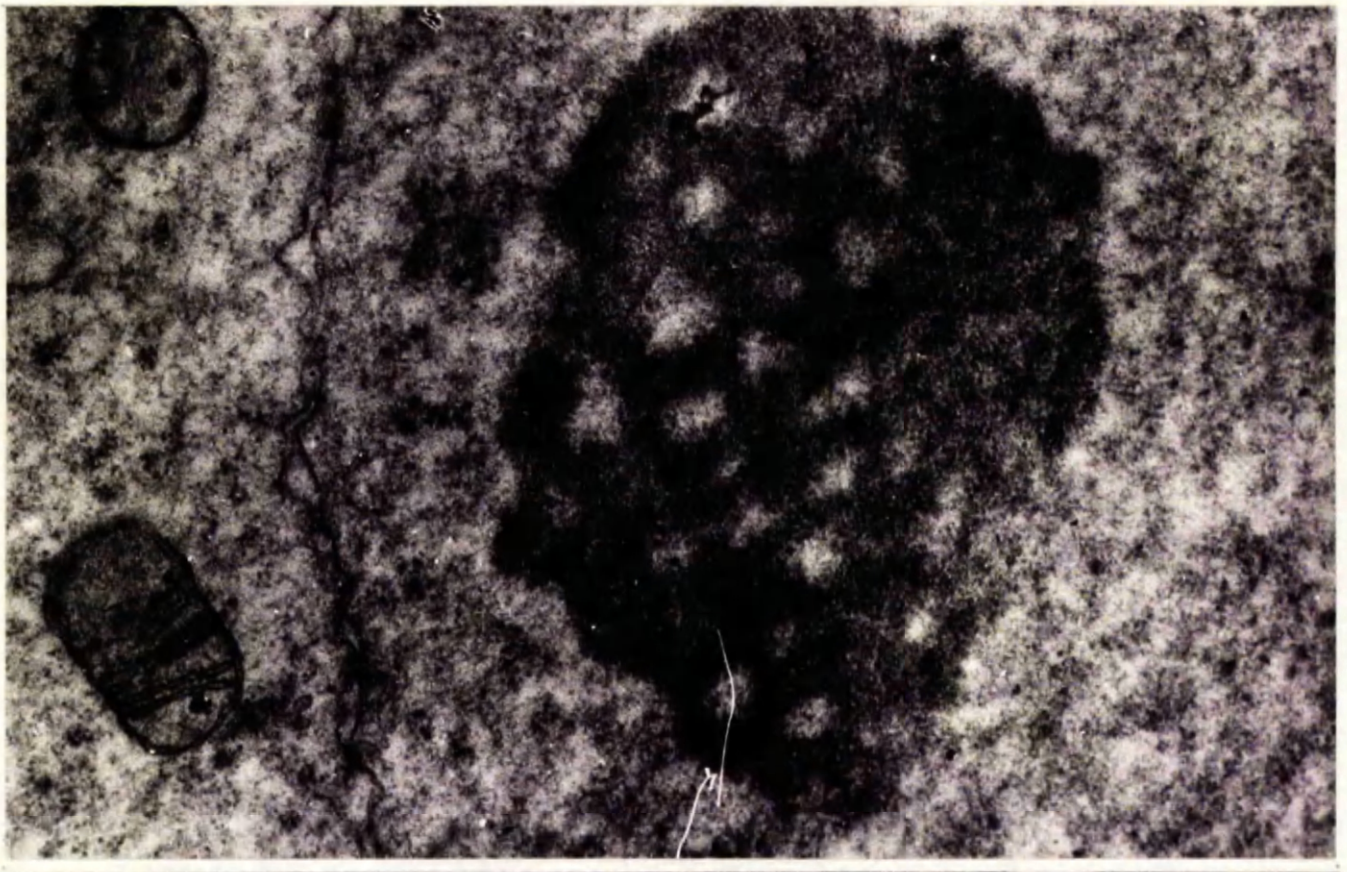
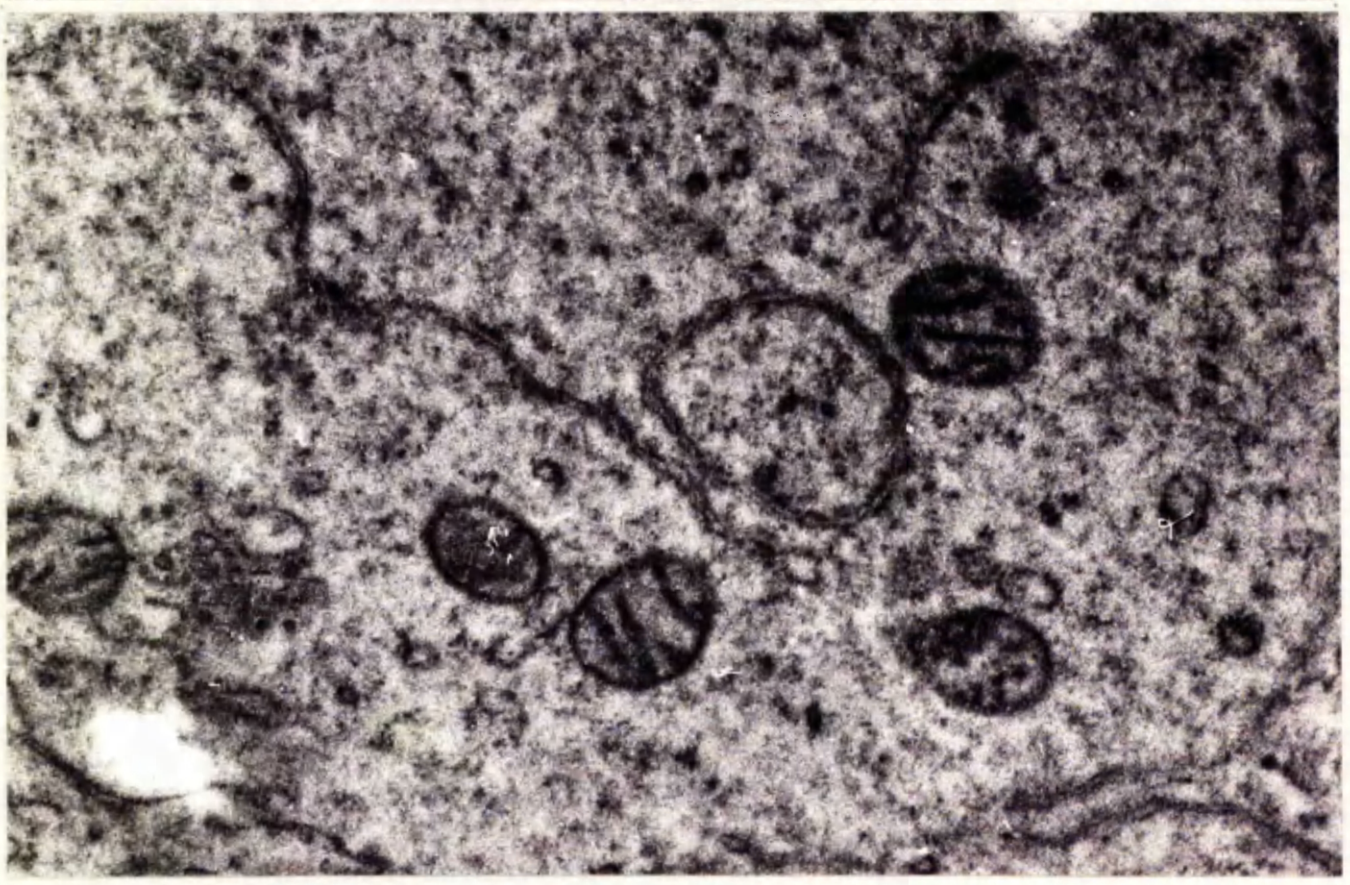


Plate 2



4.



5

Plate 3.

6

HISTOCHEMICAL STUDIES OF THE MOUSE TESTIS FOLLOWING COLD EXPOSURE.

Scot. med. J. (1961), 6, 6 - 11.

After the onset of sexual maturity, periodicity is a salient characteristic of reproductive processes, being manifest in the annual breeding cycle, which affects both sexes equally, and also in the succession of oestrous cycles peculiar to the female. The outward signs of seasonal periodicity in the male, such as variations in the plumage of birds and in the antlers of deer, are widely recognized to reflect rhythmic alterations in the hormonal activity of the testis. This cycle depends for its timing on an internal physiological mechanism which is influenced by environmental factors such as temperature, food, light and latitude and which is inactivated by domestication.

It has been shown (Bullough,1951) that the reproductive system of a seasonal breeder may be prematurely activated by increasing the light or the temperature during its winter period. As a logical converse of this work it seemed reasonable to assume that decreased light or environmental temperature might induce an artificial 'seasonal' regression of the reproductive system in a domestic, normally aseasonal male. Accordingly albino mice were exposed to artificial winter conditions as described below.

MATERIAL AND METHODS

Ten adult male albino mice were killed and their seminal vesicles removed and fixed in formol corrosive solution; forceps occluded the outlet of the vesicles during fixation to prevent loss of secretion by agonal contraction. The vesicles were then weighed, dehydrated in cellosolve, impregnated with ester wax, sectioned at 5μ, and stained with haematoxylin and eosin. One testis from each animal was similarly treated, some sections being stained with the McManus/Hotchkiss periodic acid-Schiff (P.A.S.) procedure (Carleton & Drury,1957), and others with haematoxylin and eosin. The other testis was fixed in formol calcium solution, impregnated with gelatin and sectioned at 10μ on the freezing microtome. Some sections were coloured with Sudan black to demonstrate total lipids present, others were subjected to Hayes' modification of Fuelgen and Voit's plasmal reaction (Lillie,1954) to show acetal phosphatides and possibly steroids (Dempsey,1948). Testicular volume was calculated on the latter testis using the formula: $V = {}^{4}/_{3}\pi b^{2}a$ where V is the volume, b half the equatorial diameter and a half the polar diameter of the testis.

In the mouse (Baillie,1960) all mature Leydig cells have abundant sudanophilic cytoplasmic lipids; a large

number of random sections of testes coloured in this fashion were projected by microscope on to paper, when the areas of black tissue were delineated by pen; a figure expressing the Leydig tissue as a percentage of the entire organ was derived by weighing the total paper field and, later, the cut-out areas representing Leydig tissue. Similarly a figure expressing the Schiff-positive Leydig tissue as a percentage of the testis was calculated. Lastly the total intertubular tissue was calculated on P.A.S. stained material, the results again being expressed as a percentage. This established controls for the project.

Twelve similar mice were weighed and confined in a refrigerator at -3°C. Caging, feeding and lighting arrangements were identical with those used for the controls. Water was provided in open dishes, enabling the animals to lick or gnaw the resultant ice. Since exposure to cold in this fashion results in an 80 per cent mortality rate in the first 48 hours unless nesting material is available (Barnett,1956), cotton wool was supplied; there were no fatalities. The mice were weighed and killed in pairs after living for 1,2,4,6,8 or 10 weeks in the severe cold. The testes and seminal vesicles were dealt with in the same manner as those from the control animals.

RESULTS

Seminal vesicle. There is a marked loss of turgor and reduction in seminal vesicle weight during the first 6 weeks of exposure to cold. Thereafter, over the ensuing 4 weeks there is a very slight rise which may be ascribed either to individual variation, or possibly represent some reversion of the previous trend (Fig.1, Table 1). Microscopic examination suggests that the diminished turgor and weight loss are principally due to a reduction in the amount of secretion stored in the organ: indeed, many of the smaller medial ramifications of the organ are seen to be totally collapsed and devoid of secretion. The histology of the epithelium and stroma is unchanged.

Testis. Haematoxylin and periodic acid-Schiff. Spermatogenesis continues normally throughout the period, although a number of abnormal seminiferous tubules make their appearance latterly (Fig. 7). Control Leydig-cell nuclei are rounded and vesicular with a fine chromatin not lining the nuclear membrane. Their abundant eosinophilic cytoplasm contains numerous minute yellow refractile granules and is bounded by a P.A.S.-positive membrane. There are a few P.A.S.-positive, diastase-resistant granules to be seen. Prolonged exposure to cold has no influence on this picture.

Table I. Effects of exposure to cold on mouse testis.

Duration of cold exposure (weeks)	0*	1†	2†	4†	6†	8†	10†
Seminal vesicle weight (mg.)	240	220	204	167	145	150	154
Intertubular tissue (% testis)	5.4	5.3	5.5	5.5	5.3	5.4	5.3
Sudanophilic Leydig tissue (% testis)	4.5	4.3	4.3	4.2	4.6	4.4	4.5
Schiff-positive Leydig tissue (% testis)	0.8	1.2	1.6	2.0	1.3	0.7	0.1
Testicular volume (mm^3)	199	198	192	190	194	190	196
Average somatic weight (g.)	48.0	48.0	47.6	47.3	47.6	46.1	46.8

*Values given are means of ten mice. †Values given are means of two mice.

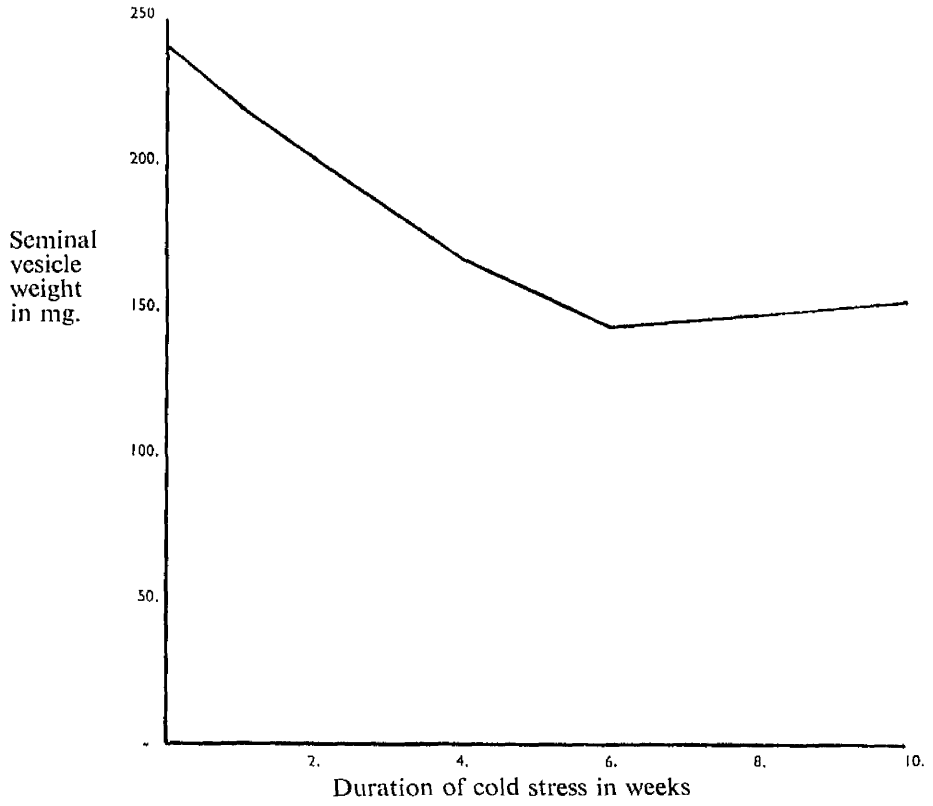


Fig. 1. Graph, as annoted.

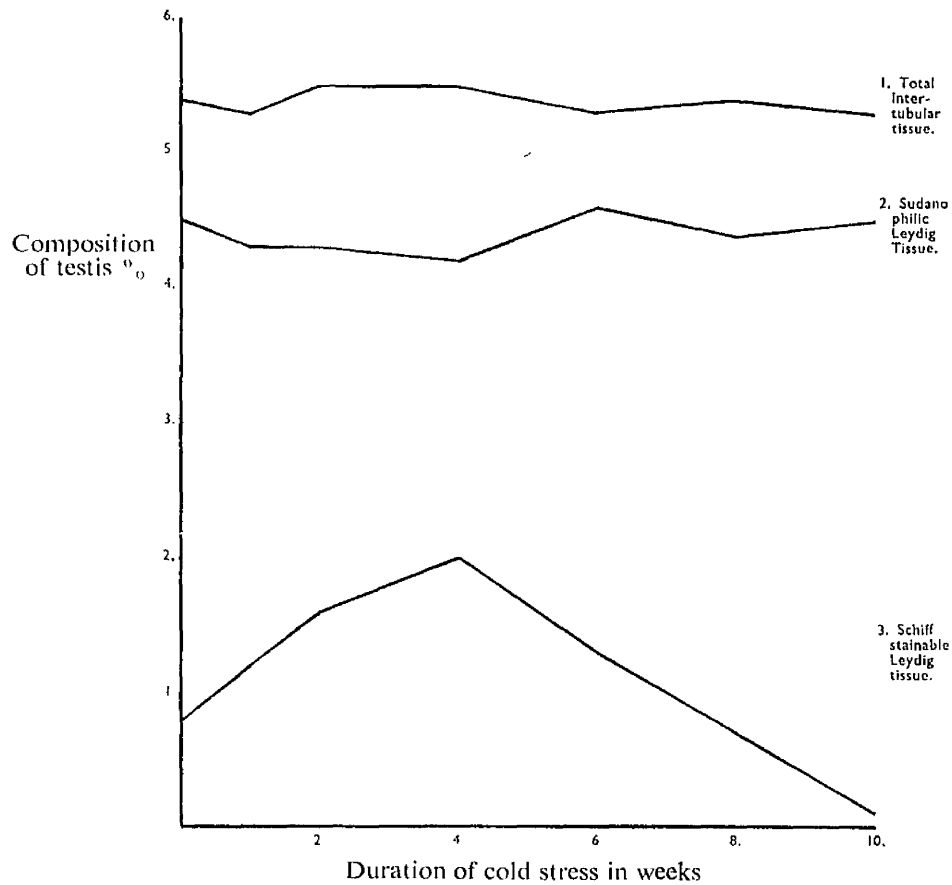


Fig. 2. Graph, as annoted.

Sudan black. All mature Leydig cells colour with Sudan black and there is no quantitative or qualitative alteration during the period of cold stress. Some abnormal tubules colour heavily with Sudan black latterly.

The plasmal reaction. Frozen sections of control testes, when exposed to Schiff's reagent present a uniform appearance (Fig.3). While the majority of Leydig cells have no demonstrable Schiff-stainable lipids a few possess abundant purple-staining lipids. On close examination these are seen to consist of swarms of minute purple granules. The Sertoli cells of the seminiferous tubules also contain droplets of Schiff-positive lipids.

During the first 6 weeks of exposure to cold the number of Leydig cells containing Schiff-positive lipids is markedly increased (Fig.4), while by contrast lipids in the Sertoli cells are reduced, being almost absent by the end of the fourth week. In the remaining 4 weeks of the experiment the Leydig cells lose all their Schiff-stainable lipids and assume a 'washed out' appearance. Sertoli Schiff-positive lipids become increasingly prominent again (Fig.5). A few abnormal seminiferous tubules appear terminally and contain masses of Schiff-positive lipids (Fig.6).

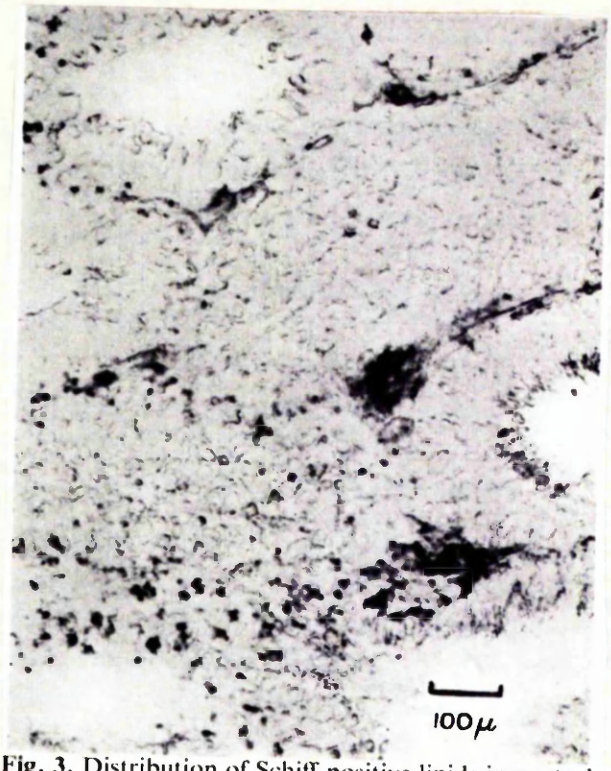


Fig. 3. Distribution of Schiff-positive lipids in control testis. Plasmal reaction.

Fig. 4. Distribution of Schiff-positive lipids in testis after 4 weeks of cold exposure. Plasmal reaction.

Fig. 5. Distribution of Schiff-positive lipids in testis after 10 weeks of cold exposure. Plasmal reaction.

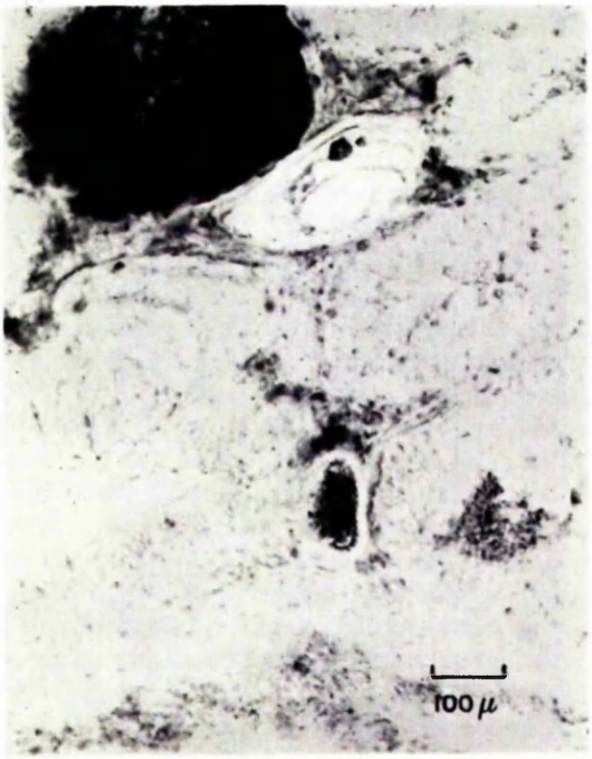


Fig. 6. An abnormal seminiferous tubule containing masses of Schiff-positive lipids after 8 weeks of cold exposure. Plasmal reaction.

Fig. 7. Abnormal seminiferous tubules after 10 weeks of cold exposure

Periodic acid-Schiff.

Quantitative observations. These are summarized in Table 1.

Since testicular volume is constant throughout the period (at 190-200 cu.mm.) the figures representing testicular composition may be viewed as an index of the total volume of Leydig tissue and of total volume of Schiff-positive Leydig tissue (Fig.2). The total intertubular tissue (Leydig tissue, blood vessels, mesenchyme etc.) volume is unchanged by cold stress. The total Leydig tissue volume is also unaltered, but the volume of Schiff-positive Leydig tissue shows a considerable initial increase which is maximal at the end of the fourth week: the volume of Schiff-positive Leydig tissue ultimately falls below the control level. No significant variations in systemic weight occur.

DISCUSSION

Seminal vesicle. During the first 6 weeks of exposure to cold stress the seminal vesicle weight falls by some 39 per cent. There is no histological evidence to suggest that the weight loss is in any way due to changes in the epithelium or stroma of the gland; it appears to be entirely the result of reduced secretory activity. Further, since the systemic weight is virtually unaltered the reduction in seminal vesicle size must reflect a diminished secretion of androgen in the animals concerned.

Following corticotrophin (ACTH) administration diminished androgen release was found to be followed by loss of secretory activity on the part of the seminal vesicle accompanied by few of the histological changes of castration (Baker et al., 1950). This would suggest that moderate reductions in androgen release are reflected by physiological changes in the seminal vesicle, while the drastic reductions which follow castration are reflected by anatomical as well as functional changes.

Testis. No quantitative or qualitative histological or histochemical changes are seen in sections stained with haematoxylin and eosin, the P.A.S. procedure, or with Sudan black, implying that androgen output by the testis need not be reflected either by changes in the total volume of Leydig tissue or by alterations in the carbohydrates and Sudan stainable lipids in individual cells. Baker et al. (1950) found that ACTH-induced depression of androgen release, similarly demonstrated by reduction in the seminal vesicle size, had no effect on the distribution and quantity of interstitial Sudanophilic lipids and alkaline phosphatase. While these workers do describe on the basis of their haematoxylin and Masson preparations, some reduction in Leydig cell size and number after ACTH administration, observations on the prepubertal testis (Baillie,1959) indicate that Sudan black findings should

be viewed much more seriously than the impressions afforded by routine histology.

Leydig Schiff-stainable lipids (acetal phosphatides and theoretically steroids) do show changes which may be related, in the present experiment, to the alteration in testicular hormone production. During the first 6 weeks of exposure to cold, while seminal vesicle weight was falling, Schiff-stainable lipids accumulated progressively; by contrast, in the following 4 weeks, Leydig Schiff-positive lipids were entirely lost. It has previously been suggested that androgen production in the Leydig cell involves synthesis from cholesterol or acetate, storage and liberation, the amount of stored hormone or its precursors being determined by the relative rates of synthesis and liberation (Baillie,1959). This concept would explain the changes visible in the Leydig cells of mice exposed to cold. A somewhat similar storage phenomenon of steroid hormone material is described in the adrenal cortex of the ground squirrel during hibernation (Zimny,1959).

The varying levels of Sertoli-cell lipids following exposure to cold perhaps reflect some alteration in the 'X' hormone provisionally identified as Δ^5-pregnenolone (Howard et al., 1950).

Hypophysectomy (Coombs and Marshall,1956) and the administration of prolactin (Lofts & Marshall,1956) in birds have been shown to produce disorganization and steatogenesis of the seminiferous tubules; the occasional abnormal tubules appearing latterly in the cold exposed mouse may represent a mammalian equivalent of passerine seasonal testicular regression.

One cannot on the basis of the present findings differentiate between the possibility of purely 'seasonal' gonadal regression induced artificially and the resistance phase of the adaptation syndrome since cold, as employed in the present undertaking, might be considered to constitute a systemic stimulus to which the mouse is not qualitatively and quantitatively adapted. Under conditions of stress, involution of the seminiferous epithelium occurs accompanied by atrophy of the Leydig cells (Selye,1946); this is paralleled by regressive changes in the accessory reproductive organs. While upsets in lipid metabolism have been described during the initial shock phase, the changes in testicular lipids noted above take place during the resistance phase. It may well be that the annual regression of the reproductive system in male seasonal breeders, during unfavourable conditions, merely represents a facet of the pituitary shift in hormone production following exposure to mild stress.

SUMMARY

Twelve adult male albino mice were subjected to maintained environmental temperatures of -3°C for periods of up to 10 weeks. One testis was studied using the P.A.S. technique, the other by frozen sections. Seminal vesicle weight and cytology were taken as an indicator of androgen production.

The weight of the seminal vesicle is reduced by 39 per cent during the first 6 weeks of the experiment; this reduced weight is maintained for the remaining 4 weeks, indicating decreased androgen release by the testis. No change in testicular cytology to account for this state of affairs can be seen in H. & E., P.A.S., or Sudan-black preparations. The distribution of Schiff-stainable lipids (acetal phosphatides and possibly steroids) does alter: the Leydig cell Schiff-positive lipids increase during the first 6 weeks of cold exposure, decreasing later, while the Sertoli-cell Schiff-stainable lipids decrease first, increasing later. The occurrence of abnormal seminiferous tubules is noted. These changes may represent a true, if artificial, seasonal gonadal repression, or they may merely be one facet of the adaptation syndrome.

ACKNOWLEDGEMENTS. The author is grateful to Professor G.M. Wyburn for suggesting the topic of this paper and for the research facilities provided in his department.

REFERENCES

BAILLIE, A.H. (1959). The interstitial cell of Leydig. Endeavour Essay. British Association.

BAILLIE, A.H. (1960). The biology of the Leydig cell; histochemical and histological changes following high epididymal obstruction. J. Endocr., 20, 339

BAKER, B.L. SCHAIRER, M.A. INGLE, D.J. LI, C.H. (1950). The induction of involution in the male reproductive system by treatment with androcorticotrophin. Anat.Rec., 106, 345

BARNETT, S.A. (1956). Endothermy and ectothermy in mice at -3°C. J. exp. Biol., 33, 124

BULLOUGH, W.S. (1951). Vertebrate sexual cycles. p. 27. London: Methuen's Monographs on Biological Subjects.

CARLETON, H.M. DRURY, R.A.B. (1957). Histological technique. p. 162. Oxford: University Press

COOMBS, C.J.F. MARSHALL, A.J. (1956). The effects of hypophysectomy on the internal testis rhythm in birds and mammals. J. Endocr., 13, 107

DEMPSEY, E.W. (1948). The chemical cytology of endocrine glands. Recent Progr. Hormone Res., 3, 127

HOWARD, R.P. SNIFFEN, R.C. SIMMONS, F.A. ALBRIGHT, F. (1950). Testicular deficiency: clinical and pathologic study. J. clin. Endocr., 53, 121

LILLIE, R.D. (1954). Histopathologic technic and practical histochemistry. p. 316. New York: Blakiston.

LOFTS, B. MARSHALL, A.J. (1956). The effects of prolactin administration on the internal rhythm of reproduction in male birds. J. Endocr., 13, 101.

SELYE, H. (1946). General adaptation syndrome and disease of adaptation. J. clin. Endocr., 6, 117.

ZIMNY, M.L. (1959). Histological studies of the ground squirrel adrenal following hibernation and cold exposure. Anat. Rec., 135, 279.

HISTOCHEMICAL STUDIES OF THE MOUSE TESTIS FOLLOWING COLD EXPOSURE. II

It has previously been shown,(Baillie,1961), that when adult male mice are exposed to artificial winter conditions, with reduced light and an environmental temperature maintained at -3°C, several changes can be discerned in the reproductive tract. The most prominent of these changes is a reduction in the seminal vesicle weight of some 39% over the first six weeks of exposure to cold. This weight reduction was thought to indicate decreased androgen release by the testis. At that time testicular cytology was investigated using routine haematoxylin and eosin staining, the PAS technique, Sudan black and the plasmalogen reaction. The first three of these staining reactions failed to show any change in testicular cytology. The distribution of Leydig cell Schiff positive lipids, however, does change during exposure to cold. While the seminal vesicle weight is falling Leydig cell Schiff positive lipids increase. As seminal weight loss comes to an end the Leydig cell Schiff positive lipids are progressively lost. Abnormal seminiferous tubules containing masses of lipid were found in the mouse testis during the second month of exposure to cold. A similar histological picture has been reported in birds following hypophysectomy and the administration of prolactin, (Coombs and Marshall,1956; Lofts and Marshall, 1956.) These changes considered together were thought

to represent a true, if artificial, seasonal gonadal regression, but the possibility was felt to exist that they might merely be one facet of the adaptation syndrome described by Sally in 1946.

With the advent of newer and more accurate methods for assessing androgen production in the male, together with refinements in the histochemical techniques available for demonstrating the various enzymes involved in steroid metabolism, it was felt that it might be profitable to re-investigate the problem.

MATERIALS AND METHODS

The animals used in the project were male Swiss white mice, aged from 3 to 12 months. In all one hundred and twenty mice were used, 20 as controls the remainder as experimental animals.

The experimental animals were placed in a refrigerator at -3°C. Caging, feeding and lighting arrangements were identical with those used for the controls. Water was provided in the form of ice cubes suspended by wires from the roof of the cage. Since exposure to cold in this manner results in a mortality in the region of 80% during the first two days unless nesting material is available,(Barnett,1956,)cotton wool was supplied. The animals were killed in groups of 6 at weekly intervals

over a period of 10 weeks. Thereafter the testes and seminal vesicles of the control and experimental animals were treated in an identical manner. Immediately on sacrifice the seminal vesicles from the animals in each group were bulked and the resultant tissue was divided into two parts. Each part was weighed and the amount of fructose in one and the citric acid in the other determined. Fructose estimation was conducted by the coloromotric method of Rowe, 1934. Citric acid estimation was carried out using the coloromotric method described by Speck, Moulder and Evans in 1946, and modified by Lindner and Mann in 1960.

In both of the above determinations a Hilderspecker absorptiometer was used. One testis from each animal was snap frozen in solid carbon dioxide and sectioned on a cryostat at 20 microns. The following histochemical components were studied.

(1) Cholesterol with the reaction described by Okamoto, Shimamoto and Sonoda, 1944.

(2) Arylsulphatase using the technique first described by Ruttenberg, Cohen and Seligman, 1952.

(3) 3β-hydroxysteroid dehydrogenase using the method of Wattenberg, 1958.

(4) Lipofuscin using Pearse's 1960 method.

(5) Succinic dehydrogenase by the method of

Nachlas et al,1957, but using nitro BT as the final electron acceptor.

(6) Cytochrome oxidase activity using the G-Nadi reaction of Moog,1943.

(7) Esterase activity using the substrates indoxyl acetate, naphthol AS acetate and α-naphthyl acetate.

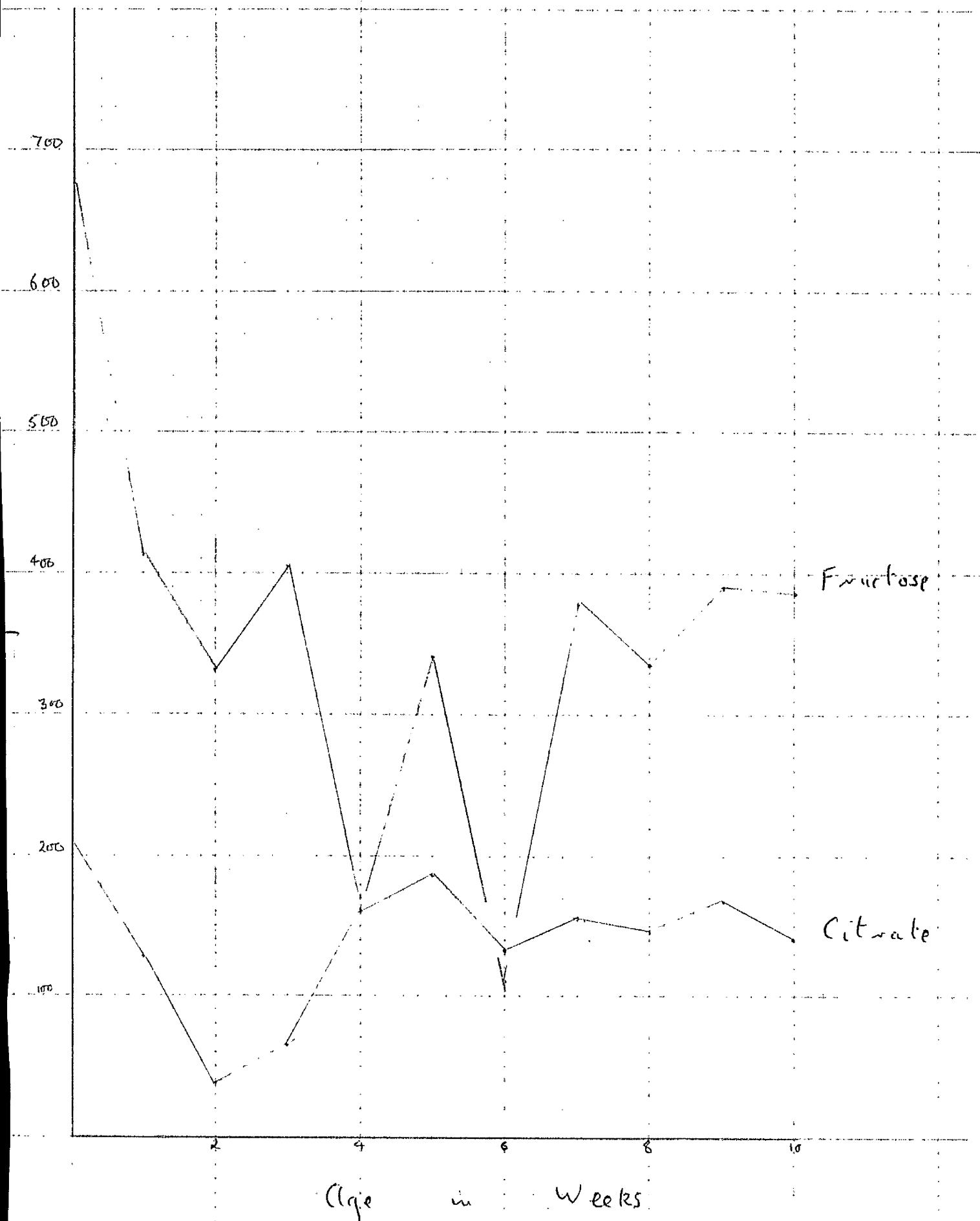
RESULTS

(1) Citric acid. The citric acid and fructose results are summarised in Table 1 and text figure 1. The average citric acid yield from the control bucks was 0.47 mg. per animal. At the end of the first week of cold stress the citric acid yield per buck had fallen to 0.28 mg. and continued to fall to 0.25 mg. at the end of the fourth week. Thereafter the average citric acid yield fluctuated between 0.27 and 0.40 mg. per animal. The concentration of citric acid per 100 grams of seminal vesicle tissue was found to be 208 mg. in the control animals. Following exposure to cold stress the concentration per 100 grams of seminal vesicle tissue fell over the first fortnight to 39 mg. per 100 grams. Thereafter the concentration fluctuated between 64 mg. per 100 grams and 169 mg. per 100 grams. At all stages during the exposure to cold the yield and concentration of citric acid are significantly reduced from the control levels.

TABLE 1

Duration of Experiment in Weeks	Control	1	2	3	4	5	6	7	8	9	10	weeks
Deaths %	-	2.5	5.7	3.2	0	4.2	20	25	0	12.5	16	%
Citrate: Yield per animal	0.47	0.28	0.25	0.31	0.25	0.40	0.40	0.35	0.32	0.45	0.27	mg.
: Concentration per 100 g.	208	128	39	64	160	186	131	155	145	169	140	mg/100 g.
Fructose: Yield per animal	1.32	0.975	0.51	0.69	0.468	0.73	0.56	0.93	0.65	0.94	0.93	mg.
: Concentration per 100 g.	675	411	331	404	158	341	110	379	336	390	385	mg/100 g.

Text - fig. 1.


700
600
500
400
300
200
100
2
4
6
8
10
Fructose
Citrate
Age in Weeks

(2) Fructose. The average fructose yield from the control animals was 1.32 mg. per buck. Following exposure to cold this average yield fell progressively during the first two weeks to reach a level of 0.51 mg. per buck and thereafter the concentration ranged between 0.46 and 0.94 mg. per buck. It will be seen from Table 1 and text figure 1 that the yield at all stages after exposure to cold is significantly below the control level. The concentration of fructose was found to be 675 mg. per 100 grams of seminal vesicle tissue and following exposure to cold this concentration fell steadily over the first six weeks to reach the lowest figure of 110 mg. per 100 grams of seminal vesicle tissue. In the remaining four weeks covered by the present data the concentration fluctuated between 386 mg. and 390 mg. per 100 grams of tissue. As with the citrate so the fructose figures both as regards yield and concentration are substantially down at all stages following exposure to cold when compared with the control levels.

(3) Cholesterol. The Leydig cells of the adult mouse testis contain abundant amounts of cholesterol. (Figure 1). Following exposure to cold stress the amount of cholesterol in the testicular interstitium becomes steadily reduced over the ten weeks studied. At the end of the tenth week most of the Leydig cells contain little

Figure 1. After one week in the freezer testicular interstitium contains abundant cholesterol. X 90

Figure 2. After 6 weeks in the freezer cholesterol deposits are much reduced. X 90.

Figure 3. After 7 weeks in the freezer lipofuscin deposits appear in the Leydig cell. X 90.

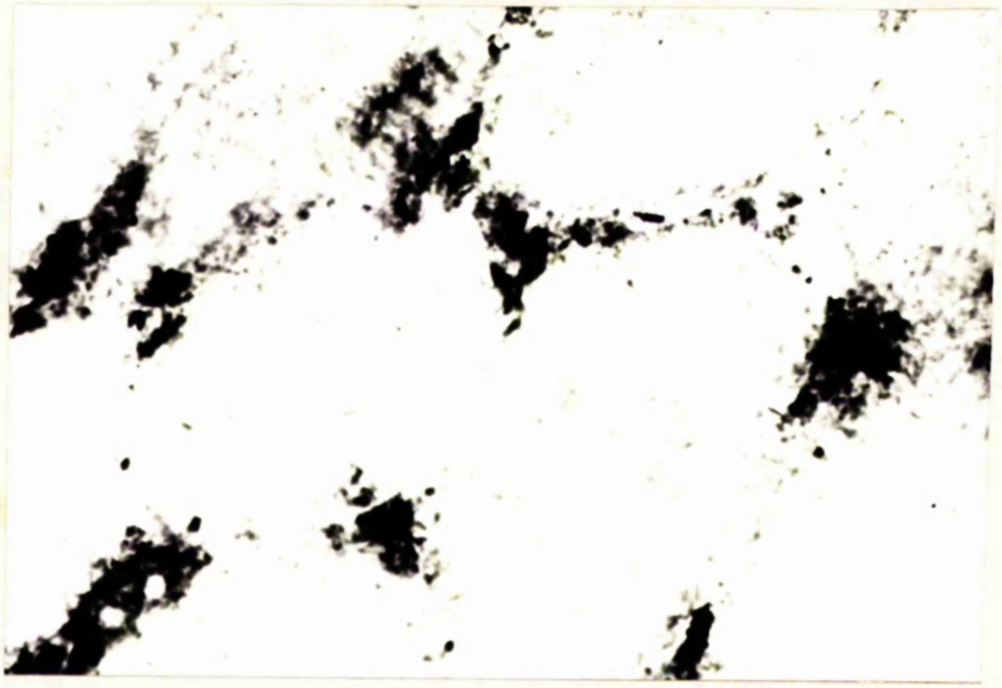


Fig. 1

Fig. 2

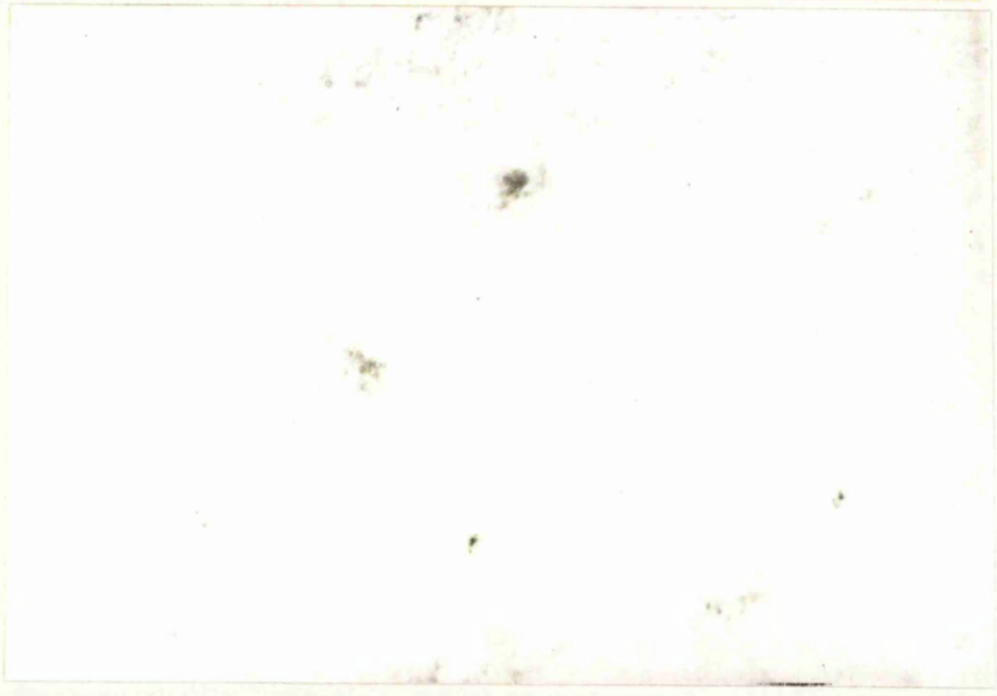


Fig. 3

or no cholesterol (Figure 2).

(4) Arylsulphatase. Arylsulphatase activity is demonstrable in the control animal and in the Leydig cells of all the experimental animals. Exposure to cold leads to a slight, but perceptible, reduction in the amount of demonstrable arylsulphatase activity.

(5) 3β-hydroxysteroid dehydrogenase. This enzyme is present in the interstitial cells of the control testes. Exposure to cold stress has no obvious effect on the distribution of the enzyme.

(6) Lipofuscin. Lipofuscin is present in a few of the interstitial cells of the control testes, but is not a prominent feature. Exposure to cold over the first three or four weeks leads to a very marked increase in the amount of lipofuscin pigment visible in the Leydig cells. This pigment persists throughout the remainder of the period studied.

(7) Succinic dehydrogenase. Succinic dehydrogenase is present in the Leydig cells of all the control testes. Following exposure to cold succinic dehydrogenase activity becomes progressively reduced so that by the end of the tenth week many of the Leydig cells have no obvious succinic dehydrogenase activity.

(8) Cytochrome oxidase. As with succinic dehydrogenase this enzyme is demonstrable in most of the

Leydig cells of the control testes. Following exposure to cold this enzyme becomes progressively reduced over the ten weeks studied so that by the end of the tenth week this enzyme is no longer a prominent feature of the Leydig cell.

(9) Esterase. Esterase activity as demonstrated by indoxyl acetate, naphthol AS acetate and α-naphthyl acetate shows no obvious relation to cold stress. Fluctuations in the amount of esterase demonstrable by these three techniques do occur, but these fluctuations are not pronounced.

DISCUSSION

It has previously been shown, (Baillie, 1961) that seminal vesicle weight is reduced during the first six weeks of exposure of an adult male to cold stress. The present data indicate that the reduction in seminal vesicle weight is accompanied by a reduction in the yield and concentration of both citric acid and fructose. The substantial reductions in these three things, namely seminal vesicle weight, citric acid and fructose concentrations, indicate beyond all doubt that androgen production is seriously reduced in the male following cold exposure. This reduction in androgen production is paralleled by a progressive fall in the amount of

cholesterol visible in the Leydig cells and by a progressive reduction in the amount of succinic dehydrogenase and cytochrome oxidase enzymatic activity demonstrable using histochemical means.

From the foregoing it seems reasonable to conclude that abundant succinic dehydrogenase and cytochrome oxidase activities together with the storage of large amounts of cholesterol in the Leydig cells of the testis indicate that these cells are producing steroids in reasonable quantities. Conversely, if these histochemical constituents are reduced in amount it would seem fair to conclude that the tissue is less active in steroid production. It has previously been suggested by Niemi and Ikonin,1962, that tissue esterases are in some way involved in steroid production. It is disappointing, however, to be unable to record any obvious relation between esterase levels and steroid production in this experiment. The constant distribution of the enzyme 3β-hydroxysteroid dehydrogenase throughout the present material is equally puzzling. The accumulation of lipofuscin pigment in the Leydig cells of the mouse following exposure to cold has a natural parallel. It is widely known that the Leydig cells of seasonal breaders accumulate lipofuscin at the end of the breading season and it is tempting to suggest that cold stress in the

circumstances described produces a true, if artificial, seasonal gonadal regression.

SUMMARY

1. One hundred adult male albino mice have been exposed to environmental temperatures of -3°C for varying periods up to ten weeks.

2. Following exposure to cold the concentration of fructose and citric acid in the seminal vesicles of the experimental animals are found to be substantially reduced at all stages.

3. Paralleling this reduction in citrate and fructose the amount of cholesterol, succinic dehydrogenase and cytochrome oxidase demonstrable in the Leydig cells of the testis are found to be reduced.

4. Lipofuscin pigment is found to increase following exposure to cold.

5. The esterase enzymes demonstrated by endoxyl acetate, naphthol AS acetate and α-naphthyl acetate do not alter significantly following cold exposure. The enzyme 3β-hydroxysteroid dehydrogenase equally does not appear to be affected by exposure to cold.

ACKNOWLEDGMENTS

The Author is grateful to Professor G.M. Wyburn for suggesting the topic of this thesis and for help and guidance at various stages of the work. Also to Professor R.J. Scothorne, lately of this Department, currently at King's College, Durham, for his help with the early part of the age series. Also to Dr. J.G. Warbrick for his help in the presentation of the latter part of the age series.

The author acknowledges gratefully the technical assistance received in the Department. Thanks are particularly due to Mr. George Marshall, Miss Mary Johnson, Mr. James McGadey, Mr. Duncan Munro and Mr. Harry Johnstone.

Lastly, but in no way least, the author is indebted to Mrs. Elizabeth Smith and Miss Margot McAllister for patiently typing this thesis.

SUMMARY

This thesis deals with some aspects of the histology, histochemistry, and ultrastructure of the mammalian testis during development and under experimental conditions.

In the interstitium of the foetal sheep testis three types of Leydig cells are discernible. The commonest contains PAS positive granules, probably glycoprotein, and Sudanophilic lipids. Schiff positive lipids are absen from those cells. Two rarer, atypical forms of interstiti cell exist. The first has groups of eosinophil granules i its cytoplasm; the second is shrunken and possesses a pycnotic nucleus. The fate of the cells containing eosinophil granules is not clear, and the cells possessing pycnotic nuclei are clearly in the process of degeneration.

The Leydig cell of the growing mouse, in common with those of other homiothermal vertebrates contains glycoprotein. In contrast to the Leydig cell of poikiloth it has no glycogen. The mouse Leydig cell has Sudanophili lipids. Lipids stainable with 2-4 dinitrophenyl hydrazine and Schiff's reagent are absent from the neonatal Leydig cell, present in large quantities in the prepubertal Leydig cell, and present in reduced amounts in the adult cell. Cytomorphosis of the Leydig cell from its mesenchymal precursor includes the acquisition of Sudanophilic lipids,

and mitochondria. Graphic representation of the growth rates of the Leydig tissue in seminal vesicles shows that both tissues grow at a similar rate, and that the growth of the Leydig tissue antedates the growth of the seminal vesicles. Mitotic figures have been demonstrated in typical Leydig cells. The actual volume of the Leydig tissue during the prepubertal phase increases at a compound rate of about fifteen per cent per day. The Leydig mitoti rate is 6.3% per day. The increment in Leydig tissue volume is thus due to cell division, plus recruitment from mesenchyme. Leydig cells in the adult testis do not appea to undergo mitosis in normal circumstances. From these facts it is plain that the concept of separate foetal and pubertal generations of Leydig cells is based on inadequate histological methods for demonstrating the relatively slowly growing Leydig tissue in a rapidly expanding prepubertal testis. The histochemical components of the Leydig cell fall into four categories.

(1) Constituents present at birth and diminishing with ag indoxyl acetate esterase and pregnenolone 3β ol-dehydrogena are examples. These perhaps reflect maternal humoral influence.

(2) Components absent at birth appearing in the first few weeks of postnatal life and increasing with age; cholestero lipofuschin, cytochrome oxidase, succinic dehydrogenase, an

α-naphthyl acetate esterase comprise this group.

(3) Elements peculiar to adult or nearly adult Leydig cells; for example, glucose, α-ketols, aryl sulphatase, and 17α-hydroxypregnenolone 3β-ol dehydrogenase.

(4) Histochemical components common to the Leydig cells of every age; for example, Sudanophilic lipids and ascorbic acid. The position of Vitamin C in the Leydig cell does not depend on either the mitochondrial location as shown by the electron microscope or on the presence of cytoplasmic interfaces. The appearance of lipofuschin in the Leydig cell is related to age, lipid droplets and PAS positive granules already noted. It seems to be closely associated with Naphthol AS-acetate esterase activity.

Using 17α-hydroxypregnenolone as a substrate, 3β-hydroxysteroid hydrogenase activity is not demonstrable in the testis of the growing mouse until the end of the tenth week of postnatal life. With pregnenolone as a substrate 3β-hydroxysteroid dehydrogenase activity is present at birth and increases progressively until the end of the sixth week of postnatal life: thereafter activity decreases over the succeeding four weeks. With DHA as substrate activity is demonstrable in all age groups betwe birth and the end of the tenth week of postnatal life and increases with age. On the basis of these findings the existence of more than one 3β-hydroxysteroid dehydrogenase

enzyme is postulated, each enzyme being substrate specific

Indoxyl acetate esterase activity in the Leydig cell prominent at birth and wanes over the first two weeks of postnatal life. Esterase activity using the substrates α-naphthyl acetate, Naphthol AS acetate, and Naphthol AS-IC acetate is first demonstrable two, three, and seven wee after birth respectively, and in each instance increases steadily with age. Differences in the volume of the acti interstitial tissue for each of these substrates are demonstrable at all ages. Naphthol AS-D acetate is only utilised by the interstitial cells of the ten week old mouse. No esterase substrate is used by all age groups, and quantitative differences exist at a given age between the individual substrates. These results are thought to imply the existence of at least five substrate specific esterases in the mouse interstitial cell, and their possible relation to steroid synthesis is considered.

Succinic dehydrogenase and cytochrome oxidase are first demonstrable histochemically in the Leydig cell at the end of the first week of postnatal life in the mouse. Using the electron microscope mitochondria have been found to be present at all stages, and show no morphological changes with advancing maturity. All three constituents increase with age; the increase is most marked during the fourth to the sixth weeks of life, and all increase at a

compound rate of roughly 12% per day. The three components are clearly closely related although some succinic dehydrogenase may have an extra mitochondrial focus. Their increase is thought to be merely an expression of Leydig tissue growth, and does not represent a true rise in cellular or mitochondrial enzyme concentrati

The ground substance of the testis of the mouse is PA positive, not metachromatic, and probably highly aggregate The basement membrane of the seminiferous tubules is intensely PAS positive, metachromatic, and not so highly aggregated. The reactivity of the ground substance to PAS reaction and toluidine blue is tentatively ascribed t the presence of chondroitin sulphate C. This compound, previously known to contain n-acetyl galactosomine glucuronic acid, tyrosine and trytophan, is associated wit arginine. The genesis of the basement membrane of the seminiferous tubule includes the formation of a sheath of atypical elongated fibroblasts, the secretion of a PAS positive metachromatic substance associated with arginine between this sheath and seminiferous rubule, the appearanc of mitochondria in the cells of the sheath, and lastly the acquisition of alkaline phosphatase by these fibroblasts a its spread to the intervening ground substance. These changes are related to the structural and nutritional requirements of the growing seminiferous tubules. In its

intense PAS positive reaction and its metachromasia the basement membrane of the seminiferous tubule resembles the ground substance adjacent to the sites of reactive protein metabolism such as growing tumours, embryonic organs, hair follicles, and skin. Using the electron microscope, between the plasma membranes of the sheath cells and those of the Sertoli cells there are three distinct zones to be found. Nearest the seminiferous tubule is a zone of decreased electron density which is thought to be specialised extracellular fluid filtered by the second or intermediate zone which consists of numerous fine particles, and the term pseudomembrane is advanced for this intermediate zone. Collagen fibrils develop in the broader outer zone adjacent to the sheath cells and fulfils the support requirements of the tubules.

After epididymal ligation regardless of the height there are two clear phases of testicular response. Initially the testis swells and the extent and duration of testicular swelling depend on the level of epididymal obstruction. A compensatory phase follows when testicular weight is nearly normal. Infarction on the other hand, leads to progressive testicular weight loss. After epididymal ligation the seminiferous tubules undergo a temporary dilation whose degree and duration is

determined by the level of obstruction. The dilatation is due initially to accumulation of fluid, later to retention of spermatazoa. Infarction results in complete and immediate collapse of the seminiferous tubules due to failure of fluid secretion. Normally both testes have the same seminiferous tubular mitotic rate. The trauma of epididymal ligation is thought to produce an immediate transient depression in the mitotic rate of the operated testis. High ligations producing marked testicular enlargement cause a gradual reduction in the mitotic rate of both the operated and controlled testi Infarction has no effect on the contralateral mitotic activity. Epididymal obstruction is followed by no chang in the absolute amount of intertubular tissue present. Testicular adaptation to epididymal obstruction is complet within fourteen days and includes a reduced mitotic rate o the seminiferous epithelium, a reduced secretion of fluid in the tubules, and the phagocytosis of shed spermatazoa b macrophages which acquire rosettes of PAS positive cytoplasmic granules. The appearance of atypical Leydig cells with masses of PAS positive material is a constant accompaniment of epididymal obstruction regardless of the site during the first fourteen days after operation.

In the neonatal mouse testis gonocytes can be discerned during the first nine or ten days after birth.

The gonocyte has ascorbic acid in its cytoplasm, but possesses no demonstrable glucose, cytochrome oxidase, succinic dehydrogenase, aryl sulphatase, 3β-hydroxysteroid dehydrogenase, or esterases. The gonocytes are shown to undergo mitosis and are thought to give rise to type A spermatogonia. Peculiar nucleolar structure is revealed by the electron microscope and seems to be characteristic of the gonocyte. The nucleolar material is very electron dense, and the nucleoli have the appearance of a sponge. The pores contain apparently typical nuclear material whic is of a much lesser electron absorption than the nucleolar material itself. Closer inspection of the nucleolar material adjacent to and overlapping the pores reveals tha the granules are in reality rather elongated electron dens bases with material of lesser electron density in the cent

Exposure of adult male mice to maintained environmental temperatures of -3°C. for periods of up to ten weeks results in certain profound changes in the reproductive tracts of these animals. The weight of the seminal vesicle is reduced by 39% during the first six weeks of the experiment, and this reduced rate is maintai for the remaining four weeks. Accompanying this reductio in seminal vesicle weight, the content of fructose and citric acid in the seminal vesicles are all found to be substantially reduced also. Paralleling this reduction

in seminal vesicle weight, citrate and fructose concentrations, the amount of cholesterol, succinic dehydrogenase, and cytochrome oxidase demonstrable in the Leydig cells are found to be reduced. Lipofuscin pigment increases with exposure to cold. The distribution of Schiff stainable lipids alters following cold exposure. The occurrence of abnormal seminiferous tubules analogous to and comparable perhaps with tubular steatogenesis seen in birds is noted. The esterase enzymes demonstrable by indoxyl acetate, Naphthol AS-acetat and α-naphthyl acetate, do not alter significantly followi cold exposure. The enzyme 3β-hydroxysteroid dehydrogenase does not appear to be affected by exposure to cold. Thes changes are thought to represent a true, if artificial, seasonal gonadal regression, but the possibility cannot be excluded that they may merely be one facet of the adaptation syndrome.