CONTRIBUTIONS TO THE BIOLOGY OF REPRODUCTION IN THE MALE ANIMAL WITH PARTICULAR REFERENCE TO THE STRUCTURE AND FUNCTION OF THE EPIDIDYMIS

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SUMMARY

This thesis is mainly concerned with a biological study of the mammalian epididymis and embraces a series of investigations that were begun in 1956.

The introduction to the thesis consists of a broad review of the subject of male reproduction. This review is at present in press for publication in the Physiology Section Series 1 - Reproductive Physiology. Ed. R. O. Greep, Medical and Technical Publishing Co., Lancaster. Chapter 9, pp. 1-57.

Then three early papers are enclosed as a prelude to the thesis and these are intended to provide background information only. The main subject of the thesis is opened with a paper on the innervation of the male reproductive tract in rabbits and in this paper the physiology of seminal emission is investigated. The work continues with an investigation of the effects of artificial cryptorchidism on epididymal spermatozoa and includes a review on the whole subject of temperature and male reproductive function. The effects of variations in scrotal temperature on blood flow in the testis and epididymis of the rat have been examined and the possible physiological significance of these effects is raised.

Factors concerned with the maintenace of viability in epididymal spermatozoa have been studied and the effects on fertilizing capacity of ageing in epididymal spermatozoa, of castration and of the administration of exogenous hormones, are also described.

The significance of the luminal environment within the

epididymis in relation to sperm maturation and sperm survival has been illustrated and the influence of circulating androgen on the chemical composition of epididymal fluid has been demonstrated. The author has speculated about the importance of the epididymal milieu in the maintenance of fertilizing capacity in the spermatozoa and in relation to their fate in the epididymis.

The thesis mostly consists of an account of experimental work carried out in the laboratory, but a major aspect of the work concerns a study of the epididymis in African testicond mammals (mammals with abdominally situated testes). These investigations have led to a new concept of the evolution of the mammalian epididymis and they represent a new approach to the biology of the organ.

Since many African mammals are seasonal breeders, the above studies have also stimulated the author's interest in the hormonal control of male reproduction in general and in the control of the testis and epididymis of seasonally breeding male mammals in particular. Most attention has been paid to the rock hyrax (Procavia capensis), but results on other species are also mentioned, especially in a paper presented to the Schering Workshop on male contraception. In this paper, the relationship of comparative studies of the epididymis to the problem of male contraception in the human being is explained.

In a later paper, the subject of the dissolution of spermatozoa in the epididymis is dealt with, and some electron microscopical findings associated with the disintegration of epididymal spermatozoa are presented.

PREFACE

The work presented in this thesis is a continuation and extension of earlier studies initiated in Cambridge in 1951. Except for three papers, which are presented as a prelude to the thesis because of their background relevance to the subject, none of the earlier work is presented, because it was carried out under supervision. Other than for these papers, only two texts that involve experiments performed in Cambridge are submitted. These papers concern work that was done after the author had completed the degree of Ph.D. in the University of Cambridge and was employed as a Demonstrator in the Department of Zoology. All other work presented was undertaken in the University of Liverpool and represents a programme of research devised and executed by the author partly with the kind assistance of colleagues, research assistants, students and technicians. The work was begun in 1956.

For the past five years, the author has been the Head and Principle Investigator of the Ford Foundation Unit of Reproductive Biology in the University of Liverpool and the Unit has published several papers that are not included here. Only those papers which concern the author's own work or where the author planned the work, directed it and actively participated in it, are presented. Thus, collaborative papers where the present author was a courtesy author only, have been omitted, together with those of his own papers where the subject is not relevant to the title of the thesis. These publications are simply listed in a separate catalogue at the beginning of the thesis.

All the work submitted has either been published or accepted for publication and manuscripts are presented in type only if they are in press or the original off-print has become faded with the passage of time. Some brief communications are also inserted but only for the purpose of continuity.

The three papers on artificial cryptorchidism in rabbits cover work which was included in a thesis for the Diploma of Fellowship of the Royal College of Veterinary Surgeons but formed only a part of the thesis. No other work in this thesis has been submitted for any other degree or diploma.

At the outset of the work, little was known of the function of the mammalian epididymis or about the details of its structure. W. C. Young (1929) had carried out experiments on sperm maturation in the epididymis of the guinea pig, and in 1923, Benoit had published his elegant investigations into the comparative histological structure of mammalian epididymides, supplementing earlier texts by Hammar (1897) on the epididymis of the dog and by Tournade (1913), von Lanz (1924, 1926) and Redenz (1924).

The present author's interest in the epididymis was stimulated by his earlier experiments on semen appraisal in rams and the effects of insulation of the scrotum on semen quality. These experiments demonstrated that when the normal thermo-regulatory function of the scrotum is upset, it is not only the spermatogenic epithelium that is damaged, but the epididymal spermatozoa as well. When insulation of the scrotum was continuous, adverse effects on semen quality were manifest in

a matter of days and after 7-9 days they were quite dramatic. But if the insulating pack was removed after 24 hrs., sperm degeneration, as revealed mainly by a striking increase in the incidence of decapitate spermatozoa in ejaculates, was not apparent for 17-23 days. Did this mean that immature spermatozoa were more sensitive to increased temperature than mature spermatozoa and that a delayed effect of 17-23 days with acute treatment represented the time of migration of damaged immature spermatozoa through the epididymis? Did it mean that with continuous treatment, the earlier onset of poor quality semen indicated a cumulative effect on mature sperms, an effect which could not occur with acute treatment? It was felt that these questions needed an answer, and that since, in an animal such as the ram at least, so many spermatozoa resided in the epididymis, consideration of the physiology of the organ was in order. Yet up to that time, it appeared that workers had addressed themselves much more to the problem of testicular function than to epididymal function. In 1957, however, after the first of the present experiments had been started, more attention was beginning to be paid to the biological significance of the epididymis, largely through the comprehensive studies of L. Nicander on the comparative histological and histochemical features of mammalian epididymides.

There could be no doubt but that the epididymis of mammals was a complex structure and perhaps this was not surprising in view of the fact that spermatozoa were known to complete their

maturation in it and needed to be retained there, sometimes for quite long periods, in a viable state. But there was no clear information about the mechanism of sperm transport through the epididymis or about the physiological principles of ejaculation. The nature of sperm maturation was controversial and in spite of some basic work on the vascularity of the testes by R. G. Harrison and E. W. Macmillan, little was known of the vasomotor response of epididymal vessels to different environmental conditions. It was known that if the testes were removed and the epididymides were allowed to remain in situ in the scrotum, epididymal spermatozoa lost their capacity for motility in vitro, but nothing was known as to how this came about, other than for the fact that the duct was androgen dependent. This had been demonstrated in the embryological studies of Dorothy Price and co-workers, and Emil Witchi and others.

Thus, the response of epididymal spermatozoa to adverse environmental conditions had never been systematically studied and the physiological control of the epididymis was far from being completely understood.

The present work was undertaken in the hope that these gaps in our knowledge might be narrowed somewhat and the work has mainly been an attempt to contribute information to the biology of the epididymis.

Recently, the author's investigations have necessitated the use of techniques for the quantitative assessment of sex

hormones by radio-immuno assay and, whilst still retaining a primary interest in the excurrent duct system of the testis, the author's attention has also been attracted to the hormonal control of spermatogenesis and Leydig cell function, particularly in relation to the problem of seasonality in male animals. It is largely for this reason, that a review, which, unfortunately is still in press, and therefore has had to be typed, is used as the introduction to the thesis. Some of the contents of this review are only barely related to the main subject of the thesis, but it is hoped that it will provide a background to the subject as a whole and afford some perspective to the main theme.

I am indebted to many people who have contributed to the progress of my work. First, I am grateful to my wife and children, for their patience, forbearance and understanding, to my parents who encouraged me to pursue a scientific career in the first place, and to those learned men who taught me how to do research when I was a young graduate student. There have been many others who have helped by their encouragement, not least of whom are those who have recommended financial support for my experiments. Then there are colleagues who have shared in the excitement of collaborative research and the research students, research assistants, post-doctoral fellows and honours students, who, by their hard work and their active minds have made my laboratory such a lively place to work in. I am immensely grateful to them all as I am to

particular technicians for their ungrudging service. I must mention especially my secretaries, namely, Mrs. Kelly and Miss Chapman, and my technician, Mrs. Boyd, whose devotion and loyalty are gladly acknowledged.

CONTENTS

SUMMARY

PREFACE (Including acknowledgements and an account of the background of the work)

INTRODUCTION

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RECENT PROGRESS IN THE STUDY OF MALE REPRODUCTIVE PHYSIOLOGY: TESTIS STIMULATION; SPERM FORMATION, TRANSPORT AND MATURATION (EPIDIDYMAL PHYSIOLOGY); SEMEN ANALYSIS, STORAGE AND ARTIFICIAL INSEMINATION

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SHORT TITLE: MALE REPRODUCTION.

RECENT PROGRESS IN THE STUDY OF MALE REPRODUCTIVE PHYSIOLOGY

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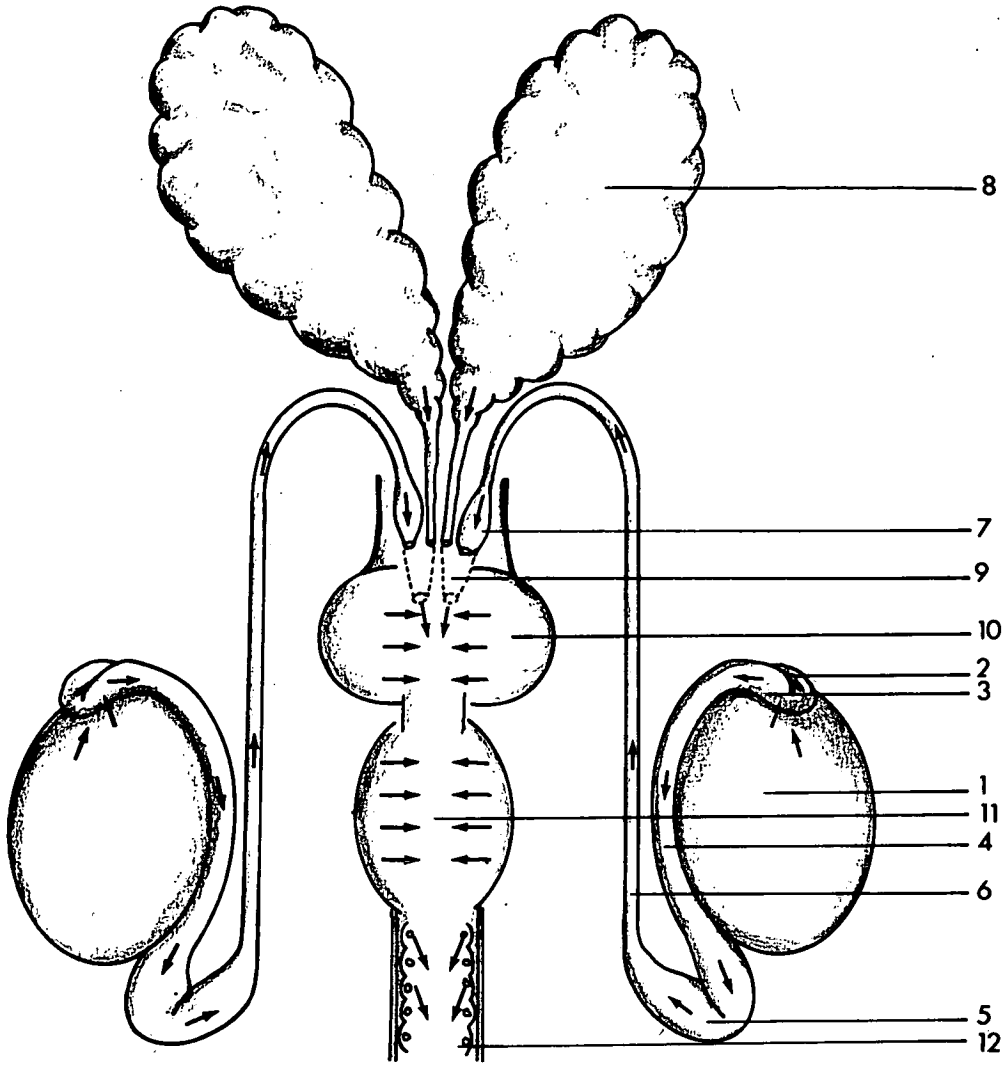
1. INTRODUCTION

To attempt a comprehensive review of the literature on male reproduction in a single chapter of a book would be unrealistic, so in the account that follows many deserving and even distinguished papers may not be cited. I, therefore, ask any authors who might feel that their work has not been fairly treated to be indulgent and understanding of the difficulties. Here, a generalized picture is presented, in the hope that some perspectives might be reached and some of the outstanding and unresolved problems might be recognized.

Essentially, the male reproductive system in mammals is designed to produce mature spermatozoa, to store them until they are needed and then to discharge them in a convenient fluid into the female so that they may ascend the female tract. For this, each male mammal has a site of sperm production, an organ for sperm maturation and storage, and a group of other accessory organs to contribute to the seminal plasma or fluid portion of the ejaculate. This is the basic theme (Text-fig. 1) although differences in the occurrence and arrangement of the organ varies widely between species.

With the evolutionary development of internal fertilization copulation has been a necessary innovation and the urge and ability to copulate are, to a great extent, due to the activity of androgens. In adult males, testosterone is the main androgen, although in some pre-pubertal mammals, androstenedione appears to predominate[1] and doubtless plays a role in sexual behaviour patterns that may be seen before puberty. There are probably many species differences, however, because in some animals the



1. Testis
2. Initial segment
3. Caput epididymidis
4. Corpus epididymidis
5. Cauda epididymidis
6. Ductus Deferens (Vas)
7. Ampulla Ductus Deferens
8. Seminal vesicle
9. Ejaculatory duct of man and some other species of mammals, but not typical
10. Prostate emptying into pelvic urethra
11. Bulbo-urethral glands
12. Other urethral glands

Figure 9.1 Generalised diagram of a mammalian male reproductive tract illustraing the direction of flow of spermatozoa and fluid

testes of the new-born are active in synthesizing testosterone[2].

Obviously, androgens are vital to male reproductive activity and they also maintain structure and function in the accessory organs. The production and release of these steroids by the testes are themselves governed by protein hormones of the adenohypophysis (gonadotrophins) and the entire process appears to be self-regulating by virtue of feed-back mechanisms. In addition, spermatogenesis, and perhaps the release of spermatozoa also, are hormonally controlled.

2. THE TESTIS

2.1 Biological aspects of gross structure and development

The mammalian testes serve both an exocrine and endocrine function, releasing spermatozoa and fluid into the seminiferous tubules and elaborating male sex hormones which pass into the blood stream.

The testes develop from the urogenital ridge and much of their tissue is mesodermal in origin. However, it is now generally accepted, after considerable controversy, that the germinal epithelium develops from "gonocytes" that migrate from the yolk sac endoderm to the site of gonadal development. The basis of the differentiation of an asexual gonad into a testis or an ovary and the manner in which the accessory organs subsequently develop are complicated subjects and it is not considered appropriate to discuss these in detail here, it is simply noted that the action of hormones or other chemical substances appears to be superimposed upon, and indeed to modify, a fundamental genetic imprint. Exactly how this happens is as yet unclear, but one point is certain, that maleness in mammals

is essentially a matter of suppressing a natural tendency by the agonadal fetus to become female[3]. At some point in the development of the mammalian embryo, differentiation of the gonad occurs and in the male, testis cords develop from peripheral epithelium, a tunica albuginea ultimately develops between the cords and the epithelium, the cords then elongate, and from this time on, development appears to proceed from the deeper parts of the gonad. Development of a testis is thus recognized by medullary development in the early gonad, in contrast to cortical development in a gonad that is destined to become an ovary.

That the seminiferous tubules eventually come to possess germ cells that are transformed into spermatogonia and that these occur alongside entirely different and highly specialized Sertoli cells, is something of an embryological mystery and it warrants more careful examination. But outside the seminiferous tubules, the interstitium, which is composed largely of the Leydig cells has quite a separate mesenchymal origin.

Development of the genital ducts follows these changes in the gonad, so that the testis comes to join the mesonephric (Wolffian) duct through efferent ductules, the seminal vesicles grow out from the mesonephric duct, and the prostate, bulbo-urethral and other urethral glands form in association with the urethra itself.

It is well known that in the majority of mammals, the testes migrate caudad and eventually come to lie outside the abdomen in a scrotal sac. Thus, testicular tissue is surrounded first by a relatively inelastic coat (the tunica

albuginea), is enclosed in a double-layered peritoneal sac (the visceral and parietal layers of tunica vaginalis) and is contained within thin scrotal skin. This skin is often pigmented, is rather sparsely endowed with characteristically coarse hairs and is always lined by a layer of connective tissue called the tunica dartos. The dartos is interesting because it contains plain muscle that allows the scrotal skin to wrinkle and when the muscle contracts it helps to bring the testes closer to the abdomen. It is a well known observation that this occurs in cold conditions when presumably temperature receptors in the scrotum are stimulated, and that the reverse is true when the environmental temperature is high. Retraction of the testes themselves is primarily accomplished, however, by the action of the striated external cremaster muscle which lies on the surface of the spermatic cord and is usually regarded in most species as being a flange of the internal abdominal oblique muscle. The smooth internal cremaster muscle that is found in the mesorchium of several species is, by contrast, probably of little functional significance.

The overall design of the scrotum and its contents indicates beyond doubt that the testes require an especially precise thermoregulation, so that when the ambient temperature rises or the individual becomes febrile, maximum heat may be lost by radiation and other means, but where circumstances demand, heat loss may also be prevented. It thus seems that the scrotum is exceptionally sensitive to changes in environmental temperature and in addition to its sensory fibres carrying normal sensations,[4]

it has been shown that they are also associated, in the ram at least, with a definite thermoregulatory reflex [5,6]. This whole subject has recently been fully reviewed by Waites[7].

The need for delicate thermoregulation of the testes is confirmed by its unique vascular architecture. In most scrotal mammals, the testicular artery is excessively coiled as it approaches the gonad[8, 9], and later it takes a superficial sub-albugineal course[9]. Coiling of the artery at a point where it is surrounded by a complex of veins allows blood flowing to the testis to be pre-cooled by a counter current heat exchange mechanism[10, 11, 7], and results in a slow, virtuallly non-pulsatile flow of blood in the testis itself[12]. The superficial course of the artery on the testis will allow the blood to be cooled further. As Setchell[11] points out, this peculiar vascular pattern depends to some extent upon the location of the testis, because in some eutherian mammals in which the testes are permanently maintained in the abdomen, the testicular artery is straight and penetrates the testis parenchyma directly[13]. The testicular artery of the rock hyrax and the elephant demonstrate this well and lend still more credance to the pampiniform plexus being associated with the thermoregulation of scrotal testes.

The testicular artery of man is relatively straight compared with that of some mammals such as the ram, and it is interesting that this corresponds to a striking variation between the two species in abdominal and scrotal temperature differences. In ruminants, the difference in temperature between the abdomen

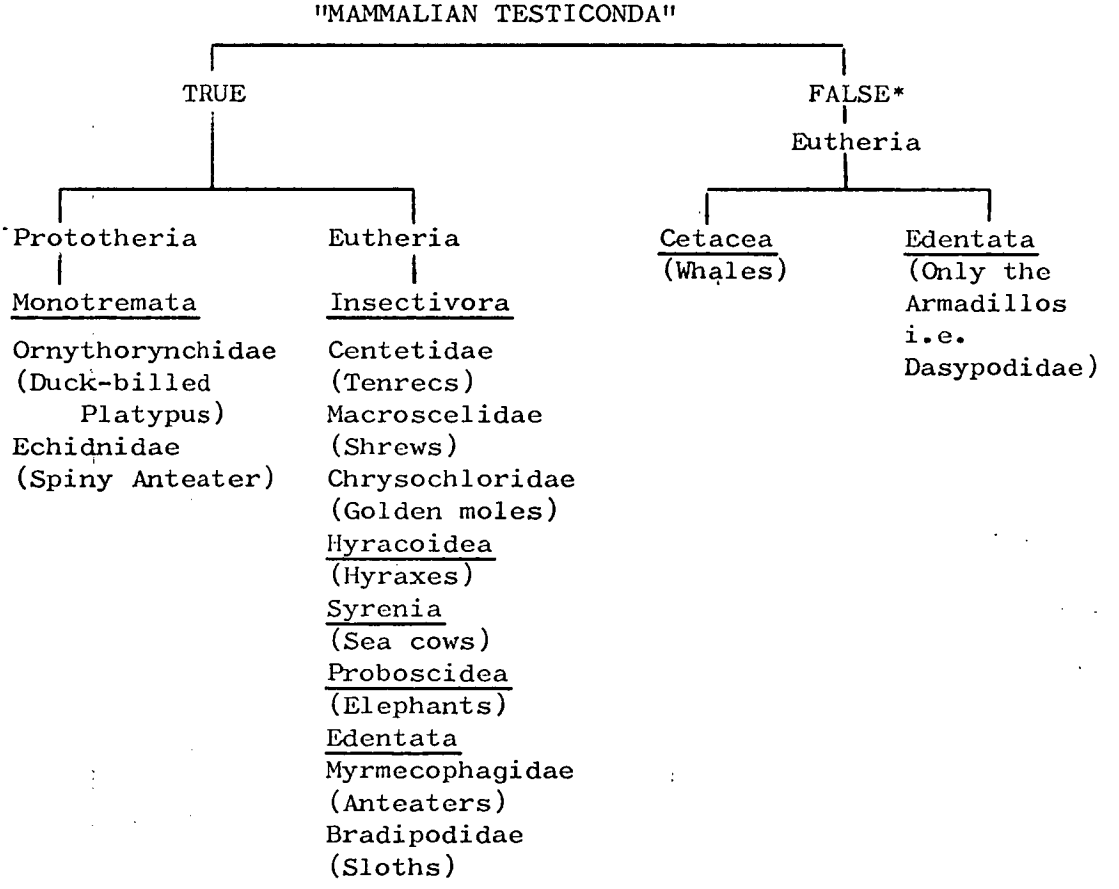
and scrotum may be as much as 8^{o} or even $13^{o}C$[8], whilst in man, it is only about $2.2^{o}C$[14]. Has the human testis become partly adapted to temperature change, therefore, or has it only just avoided being abdominal? Distinct stages in the evolution of testicular descent seem to be displayed by different species, so that in some mammals the testes are entirely abdominal and occupy the anterior part of the cavity, in others they are situated near the brim of the pelvis and yet in others they lie in the inguinal canal. It must be taken that the abdominal position of testes is the primitive condition, since it is the most common position in vertebrates and from the evolutionary standpoint scrotal testes may be regarded as being more advanced.

Owen[15] believed that inguinal testes, such as occur in the whale, represent a secondary withdrawal of the organs into the abdomen from a former scrotal position, but it is not necessary to postulate this, since an equally valid explanation is that inguinal testes represent an intermediate stage of descent. The testes of the armadillo lie just within the internal inguinal ring (personal observations) and in other edentates such as the sloths (Bradipodidae) the testes lie somewhat more anteriorally in the abdomen and might, therefore, be considered as being rather more primitive[16]. More physiological studies of the male reproductive system of mammals with abdominal testes (so-called testiconda) should be rewarding, because it is still not clear how the testes of some mammals function normally in the abdomen whilst in other species they are unable to do so.

It is also interesting that there has been considerable divergence in the evolution of testicular descent, because there are a number of eutherian mammals that, like the Prototheria, have abdominal testes and yet metatherian mammals, such as the marsupials, have scrotal testes.

On the other hand, some lagomorphs become spontaneously cryptorchid during periods of sexual quiescence, but it is likely that this is an effect of hormonal change rather than a direct cause of sterilization. It may well be, in fact, that heat has been overrated generally as a cause of sterility in animals and man.

TEXT-FIG. 2 Diagram to illustrate mammalian species with abdominal testes (so-called "testicond mammals")



*Animals where the testes are supposed to have been secondarily withdrawn (see text)

2.2. The Interstitial Tissue

2.2.1. Structural Features. The interstitial tissue of the testis consists of a rather orderly network of blood vessels and lymphatics arranged around the seminiferous tubules and a more intense and randomly orientated network of connective tissue fibres intermingled with a variety of cells including macrophages, and fibroblasts and perhaps some mast cells also. But in the majority of species, the most conspicuous interstitial cells are the Leydig cells.

The advent of electron microscopy has permitted a more detailed study of the structure of the interstitial tissue of the testis and particular attention has been focused on the Leydig cells[17,18,19,20]. These cells occur in clumps around the blood vessels, and tight junctions bind them together. The nuclei of Leydig cells are characteristically round and under the light microscope the cytoplasm has a dusty appearance. The usual organelles are present in the cytoplasm but an extensive smooth endoplasmic reticulum is striking and occurs together with an abundance of mitochondria that are possessed of tubular cristae. Various lysosomal bodies, lipid, and lipofuchsin pigment are also to be seen. In man, the Leydig cells are often seen to have a peculiar crystalloid structure in their cytoplasm[21]. This crystal, known as Reinke's crystal, occurs in the form of a hexagonal prism, and although its function is unknown, it is of interest in its specificity to man. The role of organelles in relation to Leydig cell function is not absolutely clear either,

and this applies to all species, but there is considerable evidence that the development of the smooth endoplasmic reticulum (ER) is associated with steroid biosynthesis. Hypophysectomy and LH replacement comprise the main experimental techniques that have been used in the study of this problem, which has been reviewed by Christiansen & Gillim[22].

Observations on the cytology of interstitial cells in seasonally breeding male mammals have added support to the view that smooth ER and an abundance of mitochondria are important in androgen biosynthesis, since the development of each appears to be optimal at the height of the mating season. This has been shown in the rock hyrax[23] and in the grey squirrel[24]. Towards the end of the breeding season in the hyrax, both glycogen and lipid accumulate in the Leydig cells and the smooth ER becomes vesiculated. It is possible, therefore, that steroid precursors accumulate at this time due to an arrest of androgen synthesis. Correspondingly, the testosterone content of sexually quiescent animals may be as low as 20ng as compared with 15,000ng or more during the mating season and presumably these conditions are related to the activity of gonadotrophins.

2.2.2 Pituitary control of Leydig cells. There is no firm evidence from recent work to refute the now classical contention that LH or ICSH is the pituitary hormone that controls the Leydig cells[25, 26], or that these cells are the main source of androgen production. Moreover, the Leydig cells produce more testosterone than any other steroid, at least in adults, so that although several steroids are produced by the testis[27], other androgens

and in those species where they occur, oestrogens also, are produced in much smaller quantities. Dihydrotestosterone, for instance, represents only a fraction of the total androgen in blood and much of this probably results from a breakdown of circulating testosterone[28]. This might also hold true for levels of oestradiol recorded in the blood stream[29].

Hypophysectomy results in diminished cholesterol in the Leydig cells[30] and reduced 3β-steroid dehydrogenase activity[31] and these events may represent a reduction in steroid synthesis. Reduced glucose-6-phosphate dehydrogenases also suggest relative inactivity of the cells under these circumstances, but information is still required for a full biochemical explanation of the control of Leydig cell function. It is known that there are sensitive receptors for the binding of LH or ICSH in the testis[32], that adenyl cyclase is activated[33] and it is also possible that cyclic AMP is involved in the formation of pregnenolone from cholesterol. But much hidden information remains, and it seems likely that biochemical studies at the cytological level will form a most important area of research in testicular endocrinology over the next few years. More needs to be learned, for example, about enzymatic activity in the Leydig cells.

It is also important to know precisely how different cells in the testis respond to particular hormones, because some confusion has arisen from an earlier inability to purify hormones, and particular responses may in some cases have been due to

contamination either with another exogenous hormone or with endogenous hormone. An observation might thus be the result either of a synergistic or antagonistic action of two or more hormones. It has been claimed in rabbits that FSH is a synergist to LH in the stimulation of Leydig cells[34] and in some rodents prolactin appears to be included in the control of Leydig cell function[35, 36].

If, as one hopes, purified pituitary hormones become progressively more accessible, work in this field should be made easier in the future, and a large number of puzzling problems should be resolved.

2.2.3. Androgens and pituitary regulation.

It is now accepted that androgens inhibit the release and probably the synthesis of LH, although the manner in which they do so is incompletely understood. Dihydrotestosterone (DHT) and other substances with androgenic activity can all exert this effect just as well as testosterone and so the view that androgens act on hypothalamic receptors only after their conversion to oestrogen[37] is perhaps questionable, since DHT and several other androgens cannot be aromatized. But androgens do suppress LH either directly or indirectly by inhibiting an LH releasing factor or hormone i.e. LH-RF, or LH-RH. This is a decapeptide, the action and control of which has been reviewed in some detail by Davidson[38] and by Martini[39].

It seems that, in contrast to FSH-RH, LH-RH is synthesized in the suprachiasmatic and arcuate-ventromedial areas of the brain. This was shown by hypothalamic deafferentation and

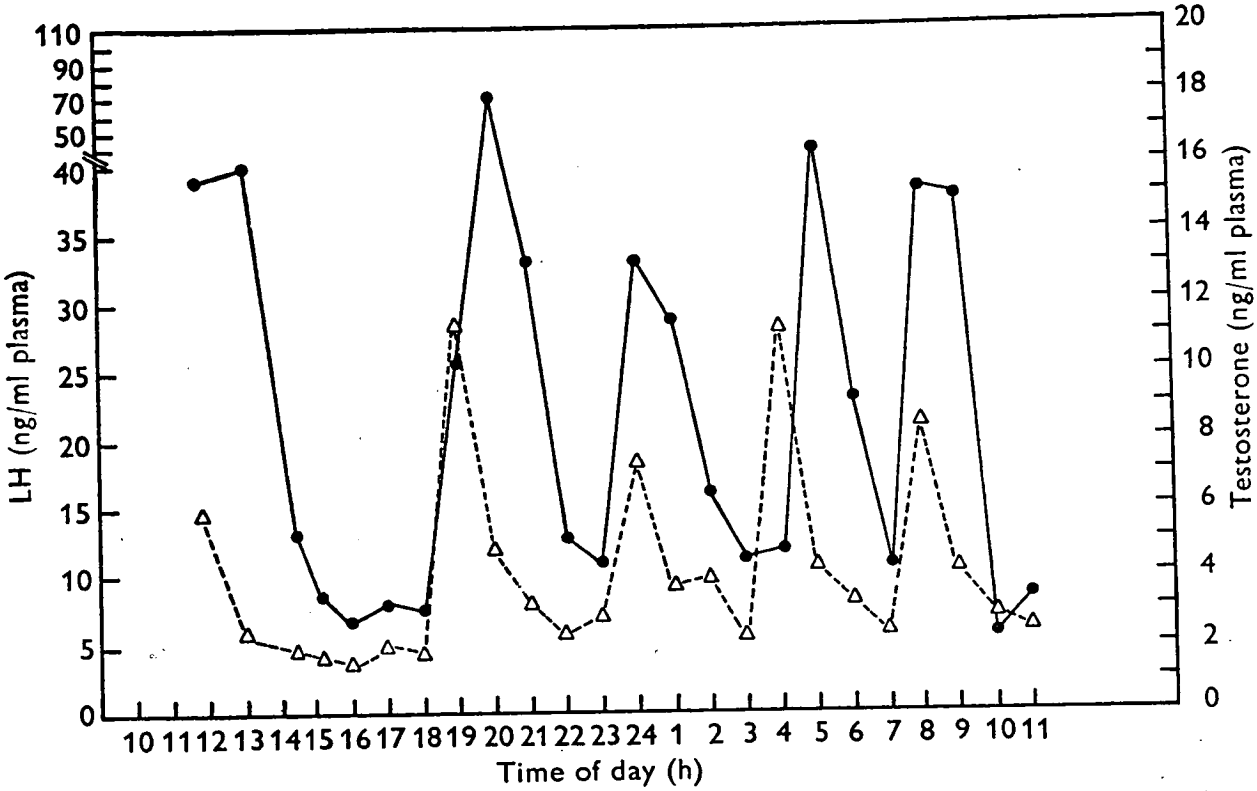


Figure 9.3 Fluctuations in the concentration of luteinising hormone (LH) (△- - -△), and testosterone (●——●) in the peripheral blood of a bull (see text). (From Katongole et al.[41], by courtesy of the Journal of Endocrinology and Cambridge University Press)

implants of protein synthesis inhibitors such as cyclohexamide[39]. The factor controls not only the release but also the synthesis of LH or ICSH and the amygdala, cortex, hippocampus and diencephalon, all appear to influence the neurones concerned in the secretion of the relasing factor. Acetylcholine might be the mediator, for Fiorindo & Martini (cited by Martini[39]) have shown that prostigmine increases LH release. But dopamine may also release LH[40], so it looks as if both cholinergic and andrenergic mechanisms play a part. Much yet needs to be done in this area, though the mechanism of LH release would seem to be simpler than that of FSH release.

One of the most interesting of recent findings in connection with this subject is that in some species LH release, in contrast to FSH release, is not constant but episodic[41, 42] (Text-fig 3). In rabbits LH levels may be increased by coitus[43] and in bulls, coitus and even the sight of a cow, stimulates the release of LH. The maximum level of LH is rapidly followed by elevated concentrations of circulating testosterone[44]. This effect is illustrated in Text-fig. 4. In rabbits, testosterone is released rhythmically, and in man there appears to be a distinct diurnal pattern of testosterone release[42].

It is evident though, from experiments on animals, that LH begins to decline before testosterone reaches its peak in the circulation. This is also apparent in seasonally breeding male mammals[45] and raises the thought as to whether testosterone feed-back only operates at a certain threshold, and, therefore, whether LH release may not be refractory to very high levels of

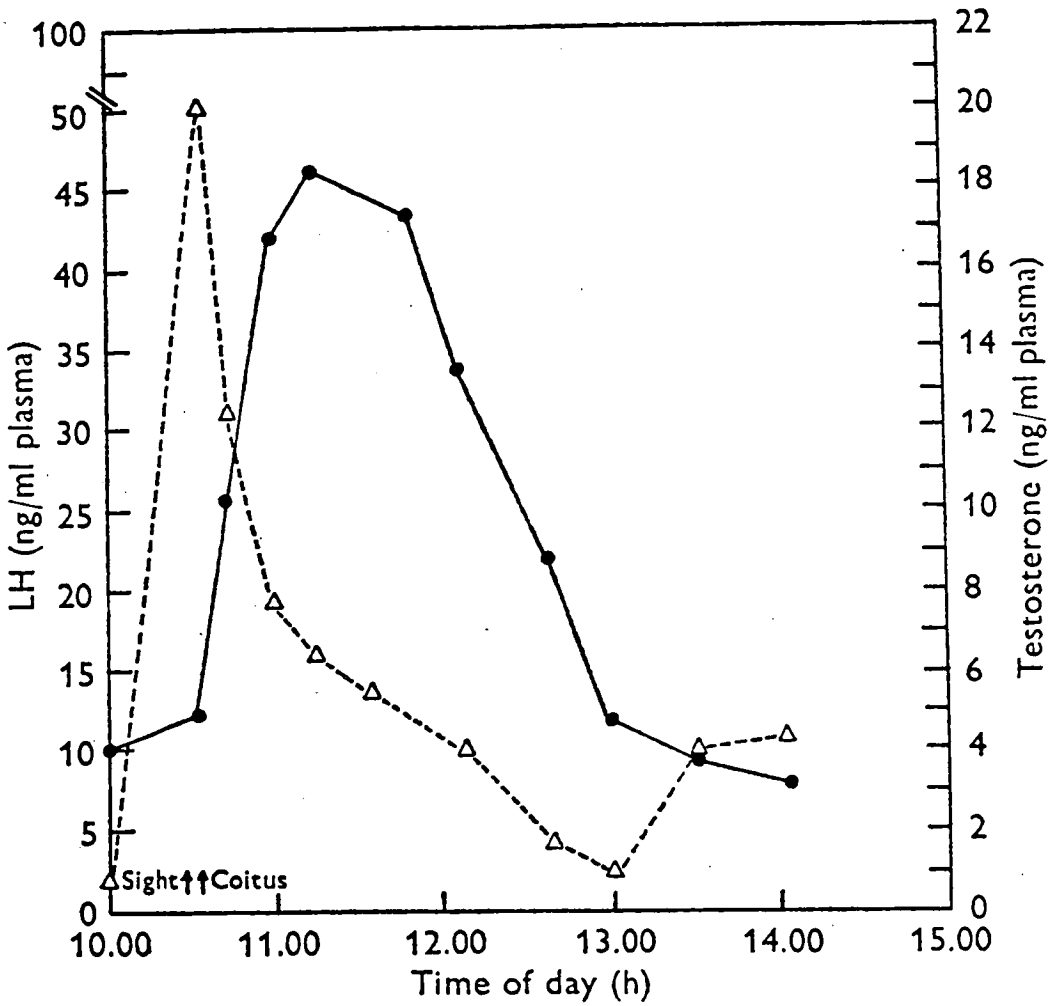


Figure 9.4 The effect of coitus on concentration of LH (△---△) and testosterone (●—●) in the peripheral blood of a bull (see text). (From Katongole et al.[41], by courtesy of the Journal of Endocrinology and Cambridge University Press)

testosterone. Here again, studies on seasonally breeding male mammals should be valuable because similar results have been obtained in a variety of species including the red deer and the roe deer[46, 47].

Finally, there is the question of whether androgens influence the synthesis and release of FSH as well as LH. This is not firmly established as a general principle and it demands further work in a variety of species.

2.3 The Hormonal Control of Spermatogenesis

Spermatogenesis consists of mitotic and meiotic activity in spermiogenesis and a phase of maturation in which spermatids are transformed into spermatozoa. This is the phase known as spermateliosis and no cellular division is involved. In the past, the study of spermatogenesis has suffered particularly from the limitations of light microscopy and from the failure of research workers to describe the process in quantitative terms.

More recently, however, a number of papers have been published on different aspects of the fine structure of germ cells[48, 49, 50, 51] and interesting descriptions of the structure and activity of the nuclei, Golgi bodies, endoplasmic reticulum and mitochondria have helped to clarify details of the process.

In addition, since the now standard works of Roosen-Runge[52], Leblond & Clermont[53] and later of Oakberg[54], it has been recognized that at certain given levels in the seminiferous tubule a number of generations of germ cells develop concurrently and that new generations are intimately related to and

synchronized with the earlier ones. Consequently, distinct cell associations occur and when such an association is complete, so that the process is ready to start again in that region of the tubule, a so-called seminiferous epithelial cycle is considered to have been completed. The stages of this cycle have been classified in a variety of ways so that Roosen-Runge & Giesel[55], described eight stages, whilst Leblond & Clermont[53], using acrosomal development as the basis of their method, described fourteen stages. Slight differences between species may exist, but recognition of the different cell associations in the cycle has permitted quantitative assessments of spermatogenic activity to be made and has thus obviated a major obstacle to accurate interpretation.

In addition to the spermatogenic cycle itself having been described, however, it has also been observed that spermatogenesis proceeds in the seminiferous tubules in a wave moving from the centre of the testis to the periphery, or in the reverse direction, and that as a result, successive periods or stages of the wave may be revealed by serial sections through the testis. Unfortunately, the duration of each stage is not constant, so that a length of tubule showing one particular stage of spermatogenesis might be much shorter than the length of tubule showing another stage. If, of course, a particular stage is especially long, several tubules as seen on an histological section may appear to be at the same stage. Cleland[56] and Hochereau[57] have also shown in rats and bulls respectively, that there is some modulation in the spermatogenic stages in any given tubule, so that any one

stage cannot automatically be expected to follow immediately a preceding stage. The position is worse in human testes[58] where cellular associations in the seminiferous tubule appear to be quite chaotic. These aberrations of the general theme make interpretation more difficult and it must also be remembered that spermatogenesis is a dynamic process and any given stage in a wave will soon move on to a subsequent stage.

Although the rate of spermatogenesis might vary, it is a continuous process except in seasonally breeding mammals, and a constant supply of cells is ensured by the production of both type A_0 and type A_1 spermatogonia, which are respectively reserve and renewing stem cells. In the rat, type A spermatogonia undergo four divisions and produce stem cell and intermediate type spermatogonia[53], whilst in the bull three generations of type A spermatogonia arise with new stem cells originating after the second one[59]. Doubtless, other species differences exist also. In any case, the total number of spermatozoa arising from these divisions is less than expected, and this is due to some degeneration of cells taking place during spermatogenesis. Very little degeneration occurs during meiosis but mostly during the division of type A spermatogonia.

Mitoses may be seen as type A spermatogonia turn into type B spermatogonia, but they are rarely to be observed in type B spermatogonia, because in these cells metaphase is of relatively short duration. The number of spermatogonia or divisions during a cycle is important in assessing spermatogenic activity

quantitively and this has beendetermined by estimating the percentage of cells that incorporate tritiated thymidine and are, therefore, likely to be synthesizing DNA[60]. The percentage of cells undergoing mitosis that also become labelled has, in addition, been used to assess gonadal divisions[60].

When type B spermatogonia divide, DNA synthesis or replication occurs in telophase and not, as might be expected, in interphase, but it takes some hours to complete and thus resting preleptotene spermatocytes have considerably less DNA than leptotene spermatocytes. This early replication is doubtless associated with the syncitium that may be seen to occur between type B spermatogonia as they give rise to spermatocytes.

Spermateliosis has been studied with the electron microscope[61, 62, 63] and among the more interesting structural changes that occur there is the extrusion of the chromatoid body from the spermatid nucleus, its contribution to the so-called ring centriole, changes in the spermatid nuclear membrane and the production of the residual body during spermiation or sperm release from the Sertoli cell[64].

The kinetics of spermatogenesis in a number of different mammals have been reviewed in detail by Courot, Hochereau-de Reviers & Ortavant[65] and by Clermont[66], and it is apparent that different generations of germ cells develop in a co-ordinated fashion to produce the characteristic cell associations seen in a spermatogenic cycle. Although it

seems likely that Sertoli cells are in some way involved in achieving this co-ordination, details of the underlying mechanism are still unknown.

Much more work is needed before spermatogenesis can be completely understood and the chemical changes taking place during the various cellular divisions stand out as needing more investigation. Already a combination of cytochemical analyses and autoradiographic studies has yielded valuable information[67]. Work of this kind has shown, for instance, that, unlike oogenesis, male meiosis is characterized by the absence of ribosomal RNA synthesis. RNA is, therefore, associated with the chromosomes at this stage of spermatogenesis and is released into the cytoplasm at diakinesis or early metaphase, thus contributing to the cytoplasmic RNA of the spermatid. Nuclear protein synthesis also ceases at the same time as meiotic RNA is eliminated from the nucleus, although an arginine-rich histone is synthesized at the end of spermiogenesis (Text-fig. 5). These events might well be extremely important factors in the regulation of nuclear activity in spermiogenesis and further work on the chemistry of chromosomal activity, particularly during the meiotic phase of spermatogenesis, is bound to be instructive.

An important development in the study of spermatogenesis is the interest taken in testicular enzymes and it may be predicted that work in this field will develop and be shown to be increasingly valuable. The subject has been concisely

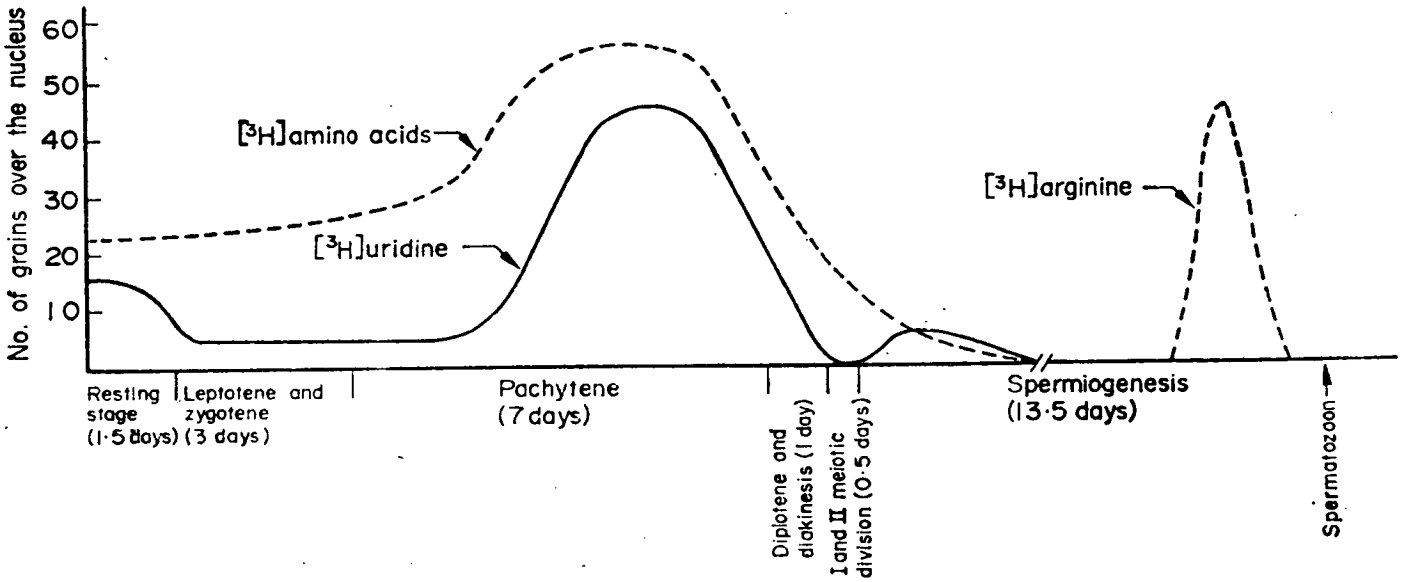


Figure 9.5 Diagrammatic description of the pattern of (^{3}H) uridine and (^{3}H) amino acids incorporation into RNA and nuclear protein, respectively, during spermatogenesis in the mouse, 1 hr after administration of the radioactive precursor. (From Monesi[67], by courtesy of the Editor, Journal of Reproduction and Fertility)

reviewed by Bishop[68] who has drawn attention to the presence of a number of enzymes in the testis including phosphatases and a variety of dehydrogenases. Some emphasis has been placed by this worker on the occurrence of sorbitol dehydrogenase in the testis and certainly this is interesting, but since its activity appears to be limited to the later stages of spermatogenesis, lactic dehydrogenase (LDH), which is active throughout the process, is probably of greater interest in relation to the control of spermatogenesis. LDH and its constituent isozymes, certainly seem worthy of closer scrutiny in relation to the spermatogenic process.

2.3.1. Pituitary hormones and spermatogenesis. A knowledge of intracellular events is ultimately necessary to an understanding of how hormones influence and control spermatogenesis. We are some way yet from such an understanding and a number of factors conspire to make the interpretation of hormonal effects difficult. First, a hormone may act differentially on different germ cells and the response of the germinal epithelium to any given hormone may differ in different species. Also, since hypophysectomy is frequently involved in studies of this subject the completeness or otherwise of the operation must be taken into account. This applies equally to the interpretation of clinical data. Nevertheless, and in spite of differing opinions, some basic tenets seem to be emerging.

The concept of FSH being the controlling factor in spermatogenesis[25, 26] has to some extent been overtaken by more recent observations. Indeed, it has been suggested that ICSH

may largely be involved in controlling the process[69] although Woods & Simpson[70], whilst confirming this, believed also that FSH may serve as a synergist to ICSH in stimulating spermatogenesis. Lostroh[71] felt that both FSH and ICSH were needed for spermatid formation in the initiation of spermatogenesis following its arrest by hypophysectomy and Berswordt-Wallrabe & Neumann[72] also felt that FSH alone was an insufficient stimulus for spermatogenesis to be restored after hypophysectomy. Also, in hypophysectomized lambs, Courot[73] has shown a synergistic effect of the two hormones, though Steinberger[74] has suggested that they may act consecutively rather than synergistically.

On the other hand, Heller & Nelson[75] found that in man, HCG was incapable of stimulating spermatogenesis and it is known that it may even be detrimental to the human testis. But, paradoxically, in the presence of some endogenous hormones, HCG may give rise to complete spermatogenic cycles[76].

FSH-rich preparations such as human menopausal gonadotrophin (HMG) have proved more successful[77] in man, as also has a combination of HMG and HCG[78], but the possibility of contamination of one hormone with another confuses the issue, and Clermont & Harvey[79] believe that the stimulatory effects of FSH are due to Leydig cell stimulation resulting from the contamination of preparations with ICSH. Using purified gonadotrophin, Courot[80] reached a similar conclusion in his studies of hypophysectomized lambs.

Undoubtedly, therefore, gonadotrophins are vital in the

control of spermatogenesis, but the specific roles of FSH and LH are not clarified in every species and it is particularly important that in man, the situation be established soon since both gonadotrophins are widely used in the treatment of male infertility. But since ICSH contamination is recognized in the most widely used preparations of gonadotrophin, it is not surprising that considerable attention has also been focused on the influence of androgens on spermatogenesis.

2.3.2. Steroids and Spermatogenesis. That testosterone can maintain spermatogenesis is now well established, but dosage is important. Thus, Moore & Price[81] illustrated that in rats, low doses of testosterone suppress spermatogenesis, and Ludwig[82] showed that this is due to pituitary suppression. When high doses of testosterone are used, however, it seems that the effects of reduced ICSH resulting from pituitary suppression are overcome by a direct effect of the androgen on spermatogenesis. Steinberger & Nelson[83] have shown that testosterone can maintain spermatogenesis in animals whose pituitary gonadotrophins have been suppressed by treatment with oestrogen and there is some evidence that spermatogenesis can actually be reinitiated by it following hypophysectomy[84]. Similarly, if spermatogenesis is partly arrested by pituitary suppression, testosterone administration may allow the process to be completed[85, 86].

In man, Heller and co-workers[87] found that testosterone may cause atrophy of the testis and these workers described the now well-known 'rebound phenomenon' following the end of treatment. But the effects of testosterone in human beings

are not comparable to those obtained in laboratory animals, because it is doubtful if it is possible to administer high enough doses to man in order to make a valid comparison. If this were not so, then surely testosterone would already be available as a contraceptive agent for the human male.

It might be that the hormonal control of spermatogenesis is mediated through the Sertoli cells in the seminiferous tubules and there is evidence that these fascinating cells are involved in some kind of hormonal activity. For some time, Sertoli cells have been considered as sources of oestrogen and Lacy[88] is of the opinion that they may also produce androgen. Some of the features of the fine structure of these cells are suggestive of steroid synthesis, and include an extensive smooth endoplasmic reticulum, numerous mitochondria and several lipid droplets surrounded by annulate lamellae. Also, lipid appears to accumulate in the Sertoli cells when spermatogenesis is inhibited and this may well represent the accumulation of steroid precursor such as cholesterol. In addition, resorption by the Sertoli cells of residual bodies (cytoplasmic masses pinched off from the spermatozoa during spermiation) is interesting, especially in view of these bodies also having a high lipid content. The function of Sertoli cells, therefore, remains a mystery and their study will be made the more difficult if they are to be found in different stages of activity in one and the same tubule. Johnsen[89], for instance, has found that in human biopsy specimens there are two types of Sertoli cells and it is evident that much still needs to be learned about them.

An important contribution to the study of the hormonal control of spermatogenesis has been made by the use of an organ culture system developed by Steinberger & Steinberger[90]. This and subsequent work has led these authors to the view that testosterone is definitely required in diakinesis following the prolonged meiotic prophase of spermatogenesis and that it is also required for reduction division in the production of secondary spermatocytes. The steroid might also be needed at other stages of spermatogenesis but at present its role here, versus that of gonadotrophins is controversial. Other hormones, some as yet unidentified, may also be involved in the control of spermatogenesis. Steinberger[74] has mentioned growth hormone, and steroids other than androgens may also play a part. Oestrogens, for example, are known to suppress spermatogenesis in the rat and hamster, exerting their action via the pituitary, whereas the inhibitory action of progestogens on spermatogenesis appears to depend upon the preparation that is used. The subject has been succinctly reviewed fairly recently by Steinberger[74].

2.3.3. Testicular Feed-back. Since testosterone exerts a regulatory effect on the synthesis and release of LH, it seems reasonable to expect that a similar feed-back mechanism associated with spermatogenesis might exist for controlling the production and release of FSH. Such a mechanism is established, for it is well known that when spermatogenesis is impaired, gonadotrophin production increases even when the Leydig cells are functioning normally.

Johnsen[91] believes that in man, spermatogenic feed-back only operates when spermatozoa are present in the seminiferous tubule and that only the last stages of spermatogenesis, including spermatids and spermatozoa, are involved in the mechanism. Moreover, this worker found a correlation between the level of gonadotrophins and the number of spermatozoa in the ejaculate, which may be regarded as supporting evidence. Paulsen[76] claimed that an increase in gonadotrophin occurring in oligospermic men was mainly due to FSH, and this hormone has been shown to accumulate in the seminiferous tubules[92]. However, de Kretzer, Burger, Fortune, Hudson, Long, Paulsen & Taft[93] found no correlation between various degrees of oligospermia and plasma FSH titres. De Kretzer (personal communication) believes that spermatozoa and spermatids are not necessary for testicular feed-back, because in many cases of impaired spermatogenesis there is damage to spermatogonia as well as to spermatids. In some cases, such spermatogonial damage may be recognised electron microscopically, but missed under the light microscope.

Exactly how the germinal epithelium exerts a regulating influence on gonadotrophins is still unknown, therefore, and requires some re-appraisal. Johnsen[91] has implicated the lipid rich residual bodies in the mechanism and put forward the hypothesis that these bodies might contain a gonadotrophin inhibitory substance, namely "inhibin". If this explanation is true then the Sertoli cells must also be involved in the

process, since Lacy[94] has shown that residual bodies are in essence phagocytosed by Sertoli cells (although this worker believes that the residual bodies induce steroidogenesis in the Sertoli cells). Even if the residual bodies are not involved in pituitary feed-back, however, the Sertoli cells could still be important in the mechanism, for their intimate association with the germ cells strongly suggests that they play some part in the control of spermatogenesis. But Setchell[95] has recorded an inhibitory factor in testicular fluid and has suggested the possibility that this may gain access to the circulation by being absorbed in the head of the epididymis.

Further work on testicular feed-back mechanisms is to be encouraged and hopefully an interest in "inhibin" will be re-kindled.

2.4 Fluid Secretion and the Blood-Testis Barrier

Knowledge of the exocrine function of the testis has been greatly enhanced by the discovery that the rete testis and seminiferous tubules may be cannulated and that fluid may be obtained therefrom. This is possible in rams[96] and rats[97] as well as in other species[98] and the procedure is illustrated in Text-fig. 6. Study of the chemical composition of fluids from the rete testis and the seminiferous tubules is made somewhat difficult, because, in spite of the tubuli recti (straight termination of the seminiferous tubules) having a valve-like arrangement as they open into the rete testis[99] there is probably interchange of fluid between the two regions.

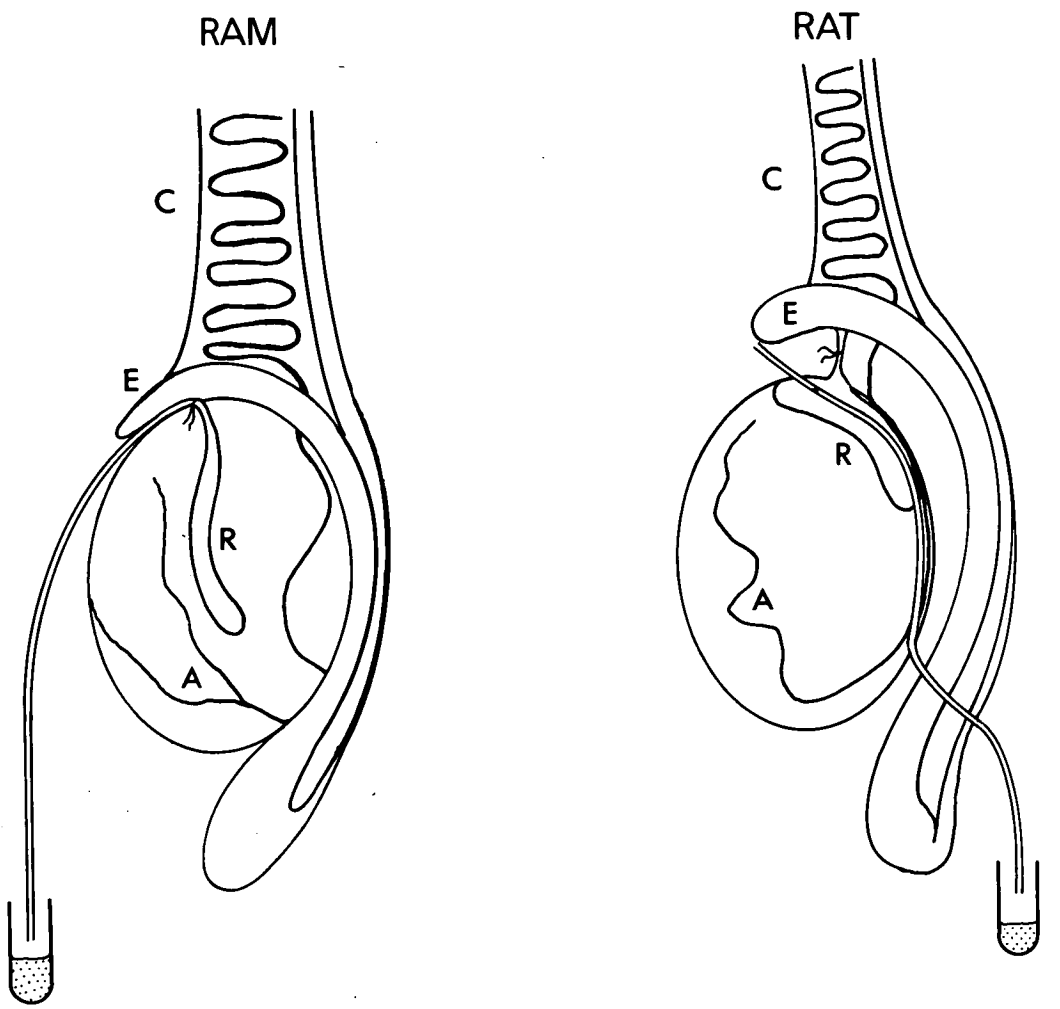


Figure 9.6 Diagrams of the anatomy of the excurrent ducts of the ram and rat and the procedures adopted for collection of rete testis fluid. R, rete testis, central in the ram and lateral in the rat; A, testicular artery; E, caput epididymidis; C, vascular cone (region of the pampiniform plexus) - in these species this comprises the coiled testicular (internal spermatic) artery surrounded by many small veins. In the ram, the caput epididymidis must be reflected and a cannula is placed in the rete testis through the efferent ducts and tied in place. In the rat, a tube with a side-hole is passed through the rete and the side-hole manoeuvred until fluid flows from the catheter (Modified from Setchell and Waites[98], by courtesy of the Editor, Journal of Reproduction and Fertility)

Nevertheless, analysis of rete testis fluid has been most informative in terms of the secretory activity of the testis, since the chemical composition of the fluid can be compared with that of blood plasma

Fluid of the rete testis has less sodium, bicarbonate, calcium and magnesium than blood plasma and it also has considerably less phosphate, but it has more potassium and more chloride.[100]

There is virtually no fructose or glucose in the rete testis fluid although there is very much more inositol and ascorbic acid than is to be found in blood plasma. There is very little citric acid or glycerylphosphorylcholine, however, and less lactate than in the blood plasma.

A number of amino acids including glycine, alanine, aspartic acid, and glutamic acid are to be found in rete testis fluid in higher concentrations than in testicular lymph or blood plasma and glutamine and asparagine can be expected to follow a similar pattern. It is suggested by Setchell[101] that these amino acids might be synthetized in the seminiferous tubules from glucose.

There are other nitrogenous compounds in the rete testis fluid also and urea is present in similar concentrations to those found in blood plasma and a number of proteins are also present. Serum proteins occur in lower concentrations than in plasma and are rather interesting in that many of them have a larger molecular weight than albumin. However, Setchell[102] described a specific protein in rete testis fluid whose molecular weight was less than that of albumin, and, in contrast, the

larger molecule of α_2 macroglobulin does not appear to pass readily into the fluid. Globulin also seems to have some difficulty. There seems, therefore, to be some selection of proteins that is not entirely related to molecular size.

Testosterone is also found in the fluid of the rete testis in fairly high concentrations but its origin and fate are as yet unknown and this and other steroids in the fluid offer themselves as interesting subjects for study in the future.

Available evidence suggests that the fluid of the rete testis is actively secreted. Among other features, there is more chloride in the fluid than can be accounted for entirely by filtration and large molecules such as inulin do not get into the fluid. Hence, the passage of substance into the seminiferous tubules and rete testis has attracted considerable attention in recent years.

Dyes do not easily penetrate the rete testis and seminiferous tubules from the blood and this lack of permeability has been studied in some detail by Kormano[103], and a blood-testis barrier comparable to the blood-brain barrier has been described by Setchell[102]. It appears that water, urea, ethanol and bicarbonate pass freely into the rete testis fluid, but that inorganic ions such as sodium, potassium, and chloride, together with creatinine and galactose pass in only slowly. Inulin and (^{51}Cr) -chromium EDTA completely fail to penetrate the tubule and permeability to albumin is low. It would seem that this permeability barrier is very much involved in the secretion of fluid by the

seminiferous tubules and there is evidence that it is influenced by gonadotrophins and by temperature[100].

Morphological studies represent a major step towards an explanation of how substances penetrate the seminiferous tubules and Clermont[104] in a study of the investments of the tubules described a layer of contractile cells on the outside of the basal lamina, being separated from it by amorphous material. A similar structure was described in the rat and mouse by Lacy & Rotblat[105] and Ross[106] respectively. The cells have been termed "interlamellar cells" by Clermont and "myoid cells" by Fawcett, Heidger & Leak[107]. The cells are characterized by an abundance of myofilaments and by several pinocytotic vesicles which occur on both the inner and outer surface of the cells. Boundaries between the cells appear to vary, so that sometimes they are short and direct whilst in others they run obliquely. Dym & Fawcett[108] have shown, however, that the extra-cellular space between myoid cells is often occluded by tight junctions, particularly when the intercellular space runs obliquely. These workers have elegantly demonstrated, moreover, that when the testis is perfused with fixative containing lanthanum nitrate, much of the lanthanum fails to penetrate the seminiferous tubules and the barrier appears to be due to the tight junctions between the myoid cells.

A second barrier exists between Sertoli cells[109]. Understandably, there are no specialized cell membrane attachments between Sertoli cells and germ cells, because presumably, desmosomes or other such specializations would prevent the

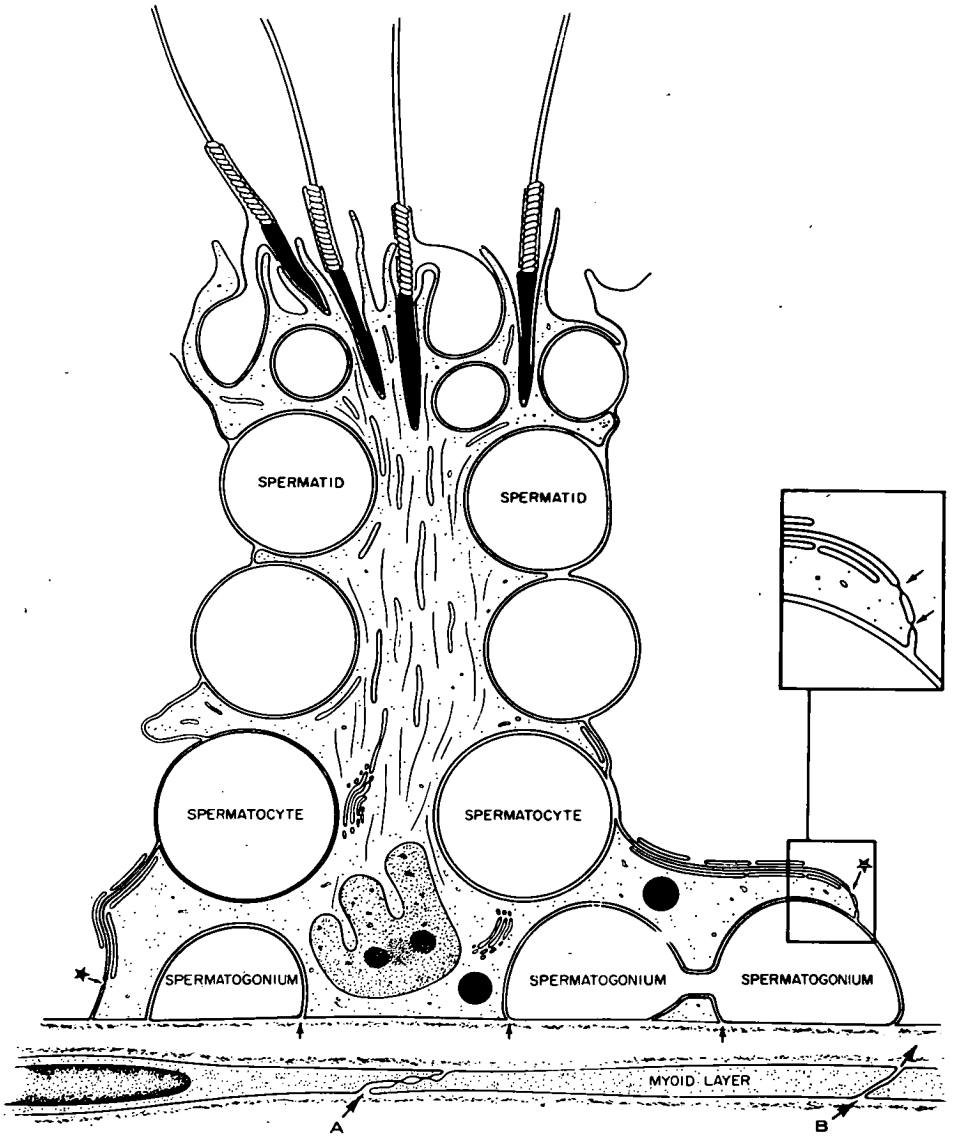


Figure 9.7 A diagram depicting the localisation of the blood-testis barrier and the compartmentalisation of the germinal epithelium by tight junctions between adjacent Sertoli cells. Note the germ cells and their relationship to a columnar Sertoli cell. The primary barrier to substances penetrating from the interstitium is the myoid layer. The majority of cell junctions in this layer are closed by a tight apposition of membranes as indicated at A. Over a small fraction of the tubule surface, the myoid junctions exhibit a 200 Å wide interspace and are therefore open as depicted at B. Material gaining access to the base of the epithelium by passing through open junctions in the myoid layer is free to enter the intercellular gap between the spermatogonia and the Sertoli cells. Deeper penetration is prevented by occluding junctions (stars) on the Sertoli-Sertoli boundaries. These tight junctions constitute a second and more effective component of the blood-testis barrier. In effect, the Sertoli cells and their tight junctions delimit a basal compartment in the germinal epithelium, containing the spermatogonia and early preleptotene spermatocytes, and an adluminal compartment, containing the spermatocytes and spermatids. Substances traversing open junctions in the myoid cell layer have direct access to cells in the basal compartment, but to reach the cells in the adluminal compartment, substances must pass through the Sertoli cells. (From Dym and Fawcett[108], by courtesy of the Editor, Biology of Reproduction and Academic Press)

constant movement of germ cells towards the lumen of the tubule[110]. In fact, it is probable that cytoplasmic projections of the Sertoli cells that extend around the spermatogonia successively retract or disappear during spermatogenesis and allow the germ cells to move luminally as they become spermatocytes.

However, special junctional complexes occur between adjacent Sertoli cells[109, 111]. Periodic narrowing of the intercellular space forms a series of gap junctions which are recognized also by bundles of fine filaments in the subjacent cytoplasm and cisternae of endoplasmic reticulum lying parallel to the cell surface. These junctions serve as a barrier to markers larger than lanthanum and occlusion is made still more effective by tight junctions that occur in addition to each gap junction or nexus. These tight junctions result in there being two distinct compartments of the seminiferous epithelium, first, the basal compartment containing spermatogonia and early leptotene spermatocytes, and secondly, the adluminal compartment with spermatocytes and spermatids (Text-fig. 7). It is apparent from this arrangement that substances which succeed in penetrating the myoid cell layer will immediately gain access to the basal compartment, but they may still be barred from reaching the adluminal compartment with its associated spermatocytes.

This fascinating subject of the blood-testis barrier requires further investigation, particularly in relation to the kinetics of spermatogenesis, the site of action of hormones and the passage of drugs into the seminiferous tubules and rete testis.

3. EPIDIDYMAL STRUCTURE AND FUNCTION

3.1 Sperm Maturation

The mammalian epididymis is an extremely long coiled duct that is attached to the testis through excurrent ducts, or ductuli efferentes, and extends over the surface of the gonad to join the ductus deferens distally. Spermatozoa must, therefore, be transported through the epididymis before they can be discharged from its distal end at ejaculation, and during periods of sexual rest, spermatozoa accumulate at this distal end which is known as the tail of the epididymis or cauda epididymidis. Not surprisingly, therefore, this region of the duct appears to have become adapted to preserve spermatozoa for fairly long periods. During their passage through the epididymis, spermatozoa undergo several changes in both structure and function which collectively are said to reflect a process of maturation. Maturation really begins in the testis because the spermatozoa spend some time in the seminiferous tubules before and during their transport into the epididymis. But testicular spermatozoa appear to be functionally inadequate and further maturation changes in both morphology and function take place in each of these independent cells as they migrate through the epididymis. These changes result in the spermatozoa acquiring the ability to fertilize an ovum if placed in the female tract and a capacity for vigorous motility if removed from the epididymis and exposed to oxygen or to a glycolizable sugar such as fructose.

Perhaps the most striking morphological change in epididymal spermatozoa is the migration of the cytoplasmic droplet (remnant

of spermatid cytoplasm) from the neck of the spermatozoon to the end of the mid-piece, an event which is accompanied by changes in the fine structure of the droplet[112, 113]. Changes in the size and shape of the acrosome have also been described in the rabbit[114] and the guinea-pig[115], although evidently these are not apparent in the human spermatozoon as it passes through the epididymis[116]. The acrosome of rabbit spermatozoa appears to contract down over the nucleus during maturation[114, 117] and increased specific gravity in the spermatozoa has also been reported[118, 119]. It would seem, therefore, that during transit through the epididymis, the spermatozoa undergo a process of dehydration. Lindahl, Kilstrom & Strom[120] also claimed that the light-reflecting capacity of the spermatozoa changes during their maturation. In addition, more disintegrated spermatozoa are found in samples taken from upper regions of the epididymis than in those from the tail of the epididymis[121, 122] and increased capacity for motility <u>in vitro</u> is a well known feature of maturing spermatozoa. Other changes, such as occur in Feulgen-positive material[123] and in the relative resistance of the spermatozoa to cold shock[124] also characterize the maturation process. Evidence that maturation of epididymal spermatozoa is accompanied by a change in their membrane characteristics was produced by Ortavant[125] and later by Glover[126] and Amann and Almquist[122] with the observation that mature and immature spermatozoa showed a difference in response to differential staining. This change is confirmed by an alteration in the electrophoretic properties and autoagglutination of the spermatozoa during their passage

through the epididymis[127, 128]. Recently, recorded differences in the binding of positively charged particles of colloidal ferric oxide to the plasma membrane of spermatozoa at different levels of the epididymis provides more evidence of a change in the cell surface during sperm maturation[129] and new techniques such as freeze fracturing for the examination of cell membranes should prove useful in further investigation of the subject.

There is little doubt that spermatozoa mature in the epididymis, but it cannot be claimed categorically that each cell does so at exactly the same rate. It has been shown in rabbits that the bulk of the spermatozoa attain their ability to fertilize by the time they reach the lower part of the body of the epididymis[130, 131, 132], but there might nevertheless be some that mature earlier or later. Even if the time of maturation is standard, it is unlikely that each spermatozoon travels through the epididymis at the same rate[133]. In order to be certain of what happens, it is important to know whether the process of maturation in the spermatozoa is entirely temporal, in which case, given a consistent and congenial environment, the spermatozoa would mature by virtue of an intrinsic ability to do so, or whether a specific or constantly changing environment is an equally or more important factor. In the past, opinions have differed about this, but more recently, Gaddum & Glover[134] put forward the view that both factors were involved in epididymal maturation, because they found that whilst spermatozoa trapped in the head of the epididymis by duct ligation were actively motile when exposed to the air, and showed some morphological signs of

maturation, their individual movement was not normal. Subsequently, Gaddum[135] has shown that the nature of movement in immature spermatozoa differs from that of mature forms, so it looks as if immature spermatozoa, blocked by epididymal occlusion, do not mature completely. This is confirmed by tests of their fertilizing capacity[136, 137]. But the site of ligation, that is whether it is low or high, does appear to be important, because very high ligation prevents any serious signs of maturation from appearing in the spermatozoa at all.

Immature spermatozoa taken from the testis, or upper parts of the epididymis of a mammal are usually either immotile or only weakly vibratile, but they can be invigorated by adjusting their environment. Thus, the addition of physiological saline may stimulate an immature spermatozoon so that it becomes actively and progressively motile. This has been seen, if not reported in print by a number of workers. It rather suggests that the environment might be the all important factor in the acquisition of progressive motility by spermatozoa but the precise relationship between motility and fertilizing capacity remains unclear. It might, in fact, be argued from existing evidence, that the development of a capacity for motility in vitro relates mainly to the environment and that its connection with sperm maturation is largely fortuitous. Clearly, the significance of motility in relation to fertilizing capacity needs to be better established. This means that more work is needed on metabolic systems in mature and immature spermatozoa.

Sperm Transport and Storage

In a wide variety of mammalian species the lumen of the tail of the epididymis is larger than that of any other area of the duct and this is mostly due to the accumulation of spermatozoa and probably fluid also, at this site. It is controversial whether the tail of the epididymis is to be regarded as a reservoir for spermatozoa, but there is no doubt that many spermatozoa are stored here even if there is some constant overspill into the ductus deferens. From the point of view of the ejaculate, spermatozoa in this storage area are very important, because unlike the rest of the duct, the tail of the epididymis contracts together with the ductus deferens in seminal emission. This is due to its sharing the same nerve supply as the ductus deferens[138]. In rabbits, stimulation of the hypogastric nerves elicits contraction of both the tail of the epididymis and the ductus deferens, but does not appear to affect the head of the epididymis or the body of the epididymis[138]. It would seem, therefore, that upper regions of the duct have their own separate autonomic innervation, although species differences are known to exist[139, 141, 140]. On anatomical and physiological grounds, therefore, the frequency of ejaculation would not necessarily be expected to influence contractile activity in the head of the epididymis or the body of the epididymis, and there is evidence in rabbits that it does not affect the rate of transport of spermatozoa through these regions of the duct[142]. This is not surprising in view of the fact that only a small part of the tail of the epididymis is emptied at ejaculation. However,

in man, the tail of the epididymis is relatively small compared with that of many other mammals and it might just be that, in contrast to these other species, frequent ejaculation in man affects the rate of sperm transport. More detailed studies on the innervation of the human epididymis would be helpful in relation to this problem, which is a rather important one because from a clinical point of view, it is helpful to know whether or not the tail of the epididymis can be completely emptied by very frequent ejaculation. In man it looks as if it is possible, but with frequent ejaculation, progressive fatigue of the seminal emission reflex must also contribute to low sperm counts. This applies also in the reverse direction so that after long periods of sexual abstinence high sperm counts may be expected, although under these circumstances, other factors, such as greater sexual excitement, give rise to general systemic effects also and these may affect the quality of the ejaculate.

But what happens to spermatozoa stored in the male tract when coitus is not taking place regularly? Many of them are probably eliminated by means of masturbation or spontaneous emissions, and doubtless some are flushed from the urethra by urine. It has, in fact, been claimed that in rams the number of spermatozoa in the urine corresponds to sperm output by the testis[143]. However, it would be unwise to generalize about all species or individuals and an accurate quantitative estimate of sperm output by the testis is, in any case, rather difficult to make, unless it is measured directly. Nevertheless,

estimates of daily sperm output in rams made by Voglmayr, Waites & Setchell[96] after cannulation of the rete testis correspond quite closely to values obtained by Lino et al.[143] from exhaustive ejaculation tests. Amann & Almquist[144], however, claimed that unwanted spermatozoa may be destroyed and absorbed in the tail of the epididymis of bulls and phagocytosis of sperms in this area has subsequently been claimed[145]. It has also been claimed that phagocytosis occurs in the human epididymis[146, 147], but there is no substantial evidence that it is associated with periods of sexual rest. The possibility cannot be entirely discarded, however, for there is evidence presented later in this chapter that massive evanescence of spermatozoa can occur when the hormonal climate of the epididymis is changed and individual hormone levels might well fluctuate considerably even in one and the same individual.

The situation in man is again obscure, but in animals it would seem that if under normal circumstances any spermatozoa are removed from the epididymis, the process is inefficient. In vasectomy there is a striking swelling of the epididymis over a period of time and in the rabbit, prolonged vasectomy results in the epididymis, especially the tail of the epididymis, occupying more space in the scrotum than the testis. In rams and rats spermatocoeles are almost inevitable following vasectomy and in the golden hamster the tail of the epididymis often ruptures altogether.

It is interesting that in man, apart from occasional spermatocoele formation, these affects do not seem to occur following vasectomy, although some clinicians have observed transitory enlargement of the epididymis. This suggests that spermatozoa and epididymal plasma are removed more efficiently from the human epididymis following vasal occlusion, or alternatively that the flow rate of fluid and spermatozoa from the human testis is much lower than that in animals. However, whether any given spermatozoon is ultimately passed out whole from the urethra during long periods of sexual rest, or is destroyed within the epididymis, it will first lose its fertilizing capacity, because the viability of spermatozoa in the tail of the epididymis cannot be preserved indefinitely[148, 149]. If the tail of the epididymis is isolated from the rest of the duct by ligation, contained spermatozoa eventually disintegrate, but even after long periods, a large number of disintegrated forms still remains in the lumen. This is further evidence that any mechanism for the removal of excess spermatozoa, if it exists at all, is not very efficient.

The disintegration of spermatozoa in the tail of the epididymis may be hastened and augmented by increasing the local temperature[150], but even then, mature spermatozoa appear to be more resistant than immature ones. In artificially cryptorchid rabbits, for instance, mature spermatozoa in the tail of the epididymis do not disintegrate until well over a week has elapsed[151] even though they lose their fertilizing capacity much more rapidly[152]. By contrast, many immature

forms are decapitate within a day of cryptorchidism.

It is interesting that in most animals the main sign of sperm degeneration is decapitation and yet in man a decapitate spermatozoon is relatively uncommon and does not appear as a characteristic outcome of increased body temperature, or local application of heat to the scrotum. In man, these conditions appear rather to evoke oligospermia[153]. Could it be, therefore, that in man, when the temperature of the scrotum is raised, mature spermatozoa are destroyed more rapidly and more completely in the epididymis than they are in most other mammals? Much more comparative information is needed on the physiological characteristics of the epididymis before such a question may be answered. Nevertheless, it seems that increased temperature curtails the survival time of epididymal spermatozoa in scrotal mammals, but it is not known yet whether the effect is directly on the spermatozoa themselves or whether it results from a heat response of the epididymal cells. There is plenty of evidence that the cells lining the epididymis play a role in the preservation of spermatozoa but their relative significance in different pathological conditions needs to be elucidated.

The Evolution and Structure of the Epididymis

The evolution of the epididymis clearly demonstrates that the duct is much more than a simple conducting tube, because it has evolved, together with the ductus deferens, from the primitive mesonephric duct. The take-over by the vertebrate testis of a duct from the urinary system must surely be significant, bearing in mind especially, that in some vertebrates

such as some of the teleosts, it has not occurred. Thus, in some teleost fishes the sperm duct is short, structurally simple and entirely separate from the urinary duct[154].

The beginnings of the take-over are to be seen in lung fishes (Diptera) where the testis joins the urinary duct through the caudal end of the kidney, but the final stages of take-over only appear to have been achieved, as in amniotes, with the advent of a metanephric kidney possessing its own excurrent duct. An intermediate position is occupied in this context by some sharks and urodeles where the metanephros, in spite of having its own duct, utilizes the mesonephric duct also. It seems, therefore, that in many primitive vertebrates, sperm storage, and sperm maturation if it occurs, take place within the testis, but that in the course of evolution, these functions have been acquired by the mesonephric duct. Thus, in the salamander, the toad (Bufo bufo) and in some snakes, spermatozoa are stored for considerable periods in ampullae or in a large central canal of the testis. In mammals, by contrast, there is no room for such storage, because the seminiferous tubules enter into a relatively small rete testis.

Recently, Glover & Nicander[155] have outlined this evolutionary trend and attempted to draw up anatomical homologies in the excurrent duct system of a variety of vertebrates. The work illustrates how the extratesticular sperm store (cauda epididymidis) has come to lie close to the testis as a result of testicular descent, since in animals with abdominal testes this sperm store

is remotely situated from the gonad. This is confirmed by observations on true testicond mammals in which the testes lie close to the kidneys[156, 13]. Moreover, although sperm ageing may simply be a continuation of sperm maturation, the two processes, as far as the bulk of the spermatozoa are concerned, take place in distinct and separate regions of the epididymis and, whilst sperm maturation is a fairly rapid process, sperm ageing is not.

Thus, whilst dynamic changes take place in spermatozoa as they traverse upper regions of the epididymis, further changes appear to be halted or restrained as the spermatozoa enter the sperm store. Such a situation demands a sophisticated system and marked regional differences in the histological structure of the epididymis indicate that a functionally complex system exists.

As many as eight distinct regions have been described in histological accounts of the epididymis, but recently it has been suggested that from a functional standpoint, the mammalian epididymis may be regarded as having three main regions, namely an initial segment, a middle segment and a terminal segment[155]. In the golden hamster, these three regions roughly correspond to the initial segment, the head and body of the epididymis combined, and the tail of the epididymis[157], but this by no means applies to every species, and failure to recognize sharply defined species differences has led to confusion. In the guinea pig, for example, the so-called body of the epididymis is, in reality, the initial segment, as structurally represented

in other species. Therefore, a terminology based on microscopical features is more meaningful than the conventional terms of head, body and tail of the epididymis, which appear to be used purely for convenience and may not refer to the same functional areas in different species.

The initial segment is the most proximal part of the epididymis and is recognized by having high epithelium with long straight stereocilia (Plates 1 & 2, figs. 1). The middle segment, according to this terminology, is complicated and consists of three main sub-regions. The first of these, consists of very vacuolated cells, whilst in the next (intermediate) part vacuoles are less profuse (Plates 1 & 2, figs. 2). In this region also, however, spermatozoa in the lumen are very concentrated (Plates 1 & 2, figs. 2). This suggests that fluid absorption occurs in the first part of the middle segment and there is plenty of supporting evidence for this. The distal part of the middle segment shows only moderate vacuolation of the cells and a lower concentration of spermatozoa in the lumen (Plates 1 & 2, figs. 3). In the terminal segment, which in most species includes the two limbs of the tail of the epididymis, the epithelium and the stereocilia are much lower than in other regions (Plates 1 & 2, figs. 4 & 5) whilst the lumen is larger and packed with spermatozoa. Most of this area serves as the sperm store.

There are four clearly defined cell types in the epididymal lining. These comprise mainly the principal cells and basal cells, but two other types may be present, namely, the so-called clear cells and halo cells. These are most apparent in the

in the epididymis of rats and hamsters, and whilst halo cells are present in the rabbit epididymis also, clear cells are not to be seen in this species.

The principal cells are the tall columnar cells that are found throughout the extent of the epididymis (Plate 3, fig. 1). They possess microvilli of variable length and type and different degrees of pinocytosis are to be seen taking place in these cells, indicating that absorption is a characteristic activity of the epididymis. Thus, pinocytotic vesicles and multi-vesicular bodies are present at most levels of the duct. Other features of the principal cells indicate that they are metabolically very active and there is a preponderance of euchromatin in lobulated nuclei and a highly developed Golgi apparatus. Lysosomes and other cell inclusions, whilst occurring variably in different species, are usually present. Mitochondria are fairly randomly distributed throughout the cytoplasm, and although rough ER is extensive, many free ribosomes are invariably to be seen in the cytoplasm also. Large dense bodies are often to be seen in a supra-nuclear position and are lysosomal[158]. Definite morphological evidence of secretion by the principal cells is so far lacking, which makes distinct areas of selective secretion difficult to define and thus strengthens the view that the epididymis is primarily an organ of absorption. Certainly, the cells of the head of the epididymis absorb both fluid and particulate matter[159, 160], but as mentioned later, chemical analyses show that some secretion also occurs.

Figure 9.8 Photomicrographs of paraffin sections of a hamster epididymis, Bouin fixation, H and E x 160 (Reduced $\frac{2}{3}$rds on reproduction)

(1) Initial segment

(2) Middle segment, intermediate part

(3) Middle segment, distal part (proximal corpus)

(4) Terminal segment, proximal part

(5) Terminal segment, distal part (middle cauda)

Arrows show a number of clear cells (see text). (From Nicander and Glover[157] by courtesy of J. Anat.

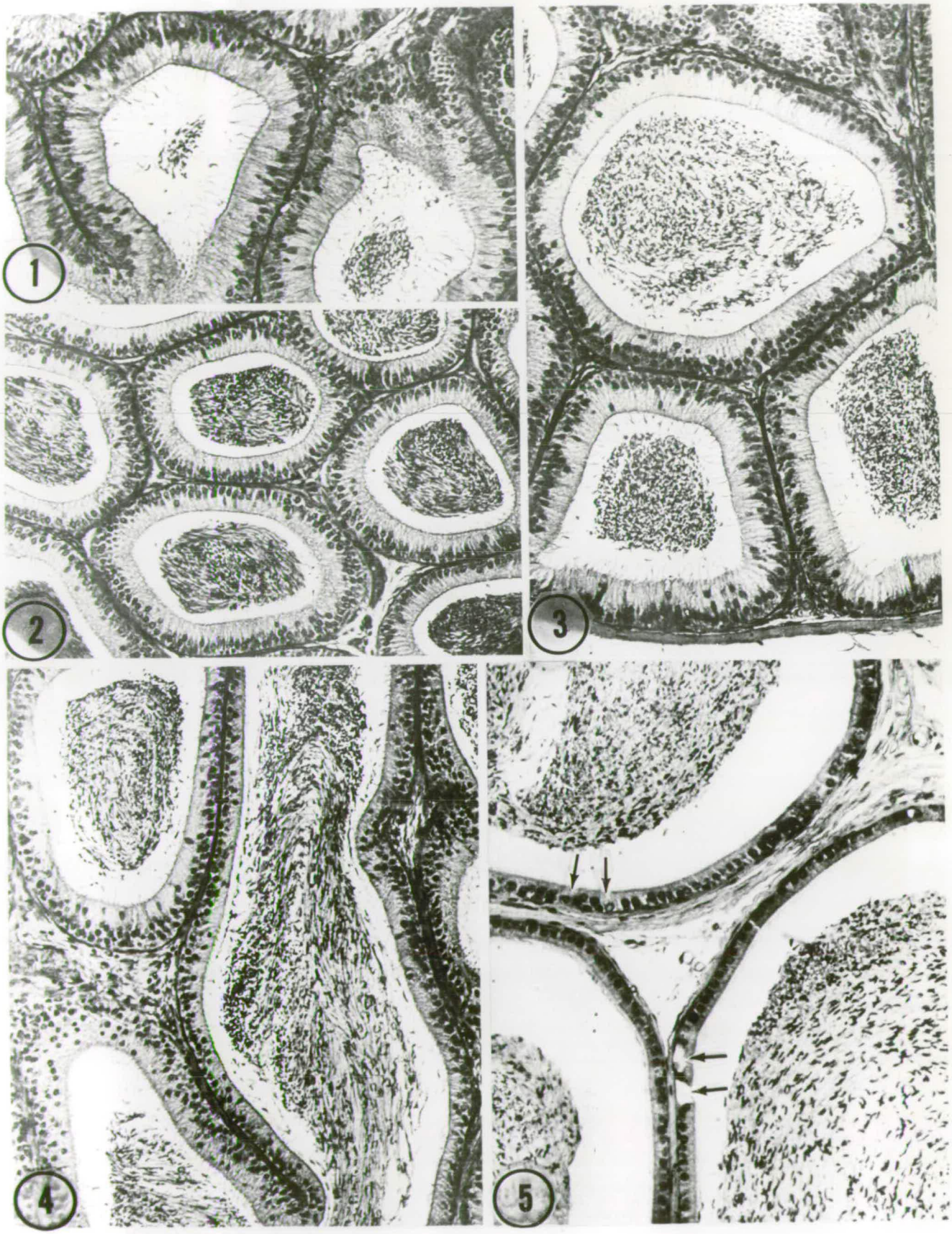

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Figure 9.9 Photomicrographs of Epon sections from a hamster epididymis. Fixed by vascular perfusion with glutaraldehyde, followed by osmium tetroxide. Toluidine blue (see text). x 1,200 (Reduced $\frac{2}{3}$rds on reproduction)

(1) Initial segment, with long, coarse stereocilia, large Golgi area (GA), columnar cell nuclei at a high level, and numerous supranuclear granules. Three small light cells (halo cells) are indicated by arrows.

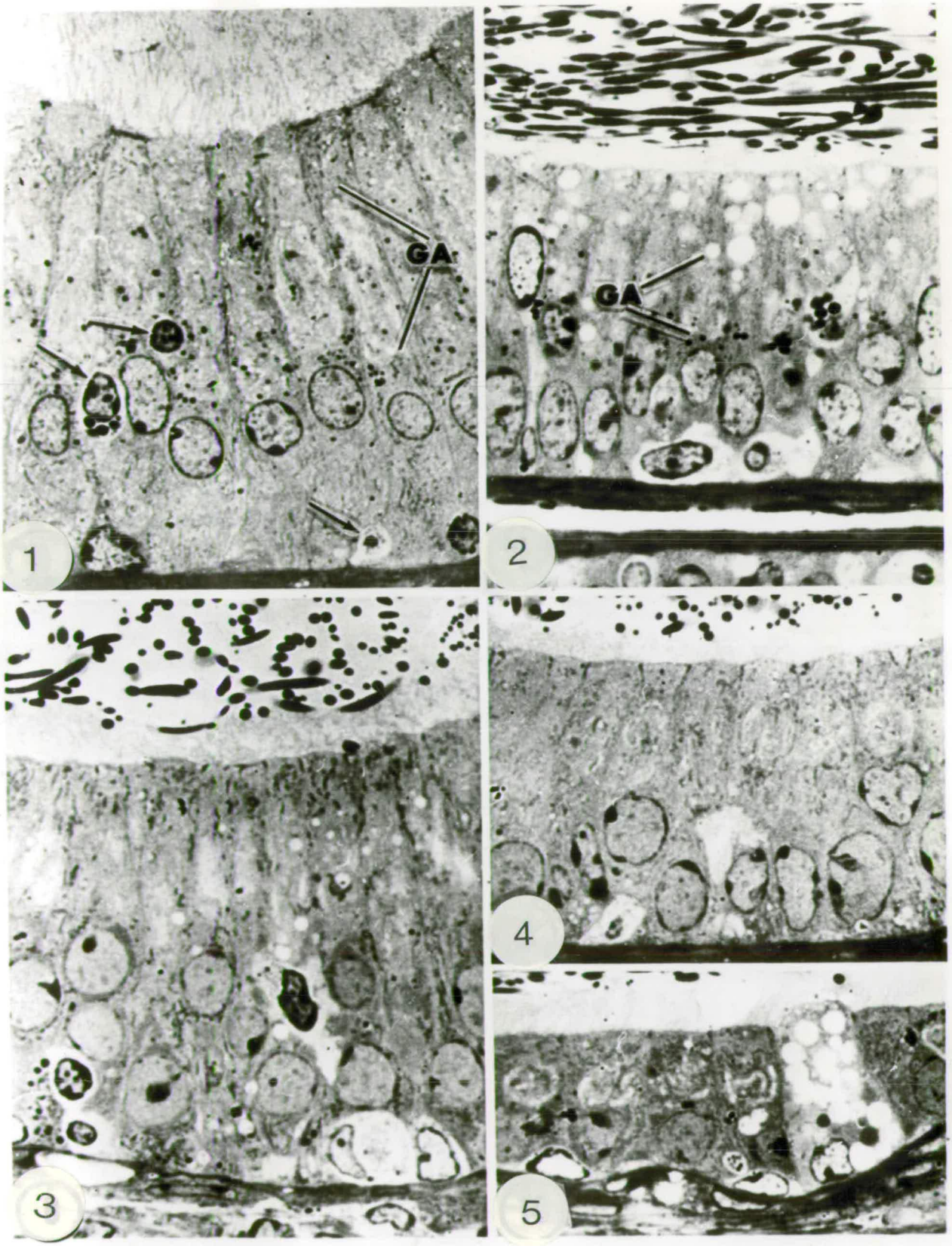
(2) Middle segment, intermediate part. Note densely crowded sperm in lumen, low stereocilia and mainly apical vacuoles. The Golgi apparatus (GA) is of moderate size.

(3) Middle segment, distal part, with few vacuoles but numerous mitochondria in apical cytoplasm. Moderate amounts of spermatozoa in the lumen.

(4) Terminal segment, most anterior part, Lower columnar cells with apparently homogeneous cytoplasm.

(5) Terminal segment, distal part (middle cauda). Low epithelium with dense cytoplasm and conspicuous Golgi area. One light, vacuolated columnar cell is also seen.

(From Nicander and Glover[157], by courtesy of the Journal of Anatomy)


GA
GA
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Apically situated tight junctions between principal cells (Plate 3, figs. 1 & 2) are probably associated with absorption of fluid, since they occur in other fluid absorbing organs such as the gall bladder. And it is also interesting that the cisternae of smooth ER are sometimes seen to form an open channel with the lumen.

Clear cells may occasionally be seen interposed between the principal cells, particularly in the terminal segment of rodent epididymides. These cells were originally named by Reid & Cleland[161] on the grounds of excessive vacuolation and they are the so-called 'holocrine cells' of Martan[162]. However, as Hamilton[163] points out, better fixation methods have now shown these cells to contain densely packed lipid droplets and a rather amorphous nucleus. Pinocytosis may be seen taking place at the basal border of these cells. They often contain supra-nuclear vacuoles and an abundance of supra-nuclear lysosomal-like bodies may also be demonstrated (Plate 3, fig. 2).

The epithelium of the epididymis is pseudostratified, but true apical cells that do not extend to the basement membrane have been described in the epididymis of the rat by Reid & Cleland[161]. These cells appear to be different from principal cells but may be their precursors. The observations of Suzuki (personal communication) on the epididymis of the rabbit and hamster indicate that apical cells actually reach the basement membrane, albeit perhaps by tenuous cytoplasmic extensions (Plate 4, fig. 1) and it would seem more likely that they are inactive clear cells.

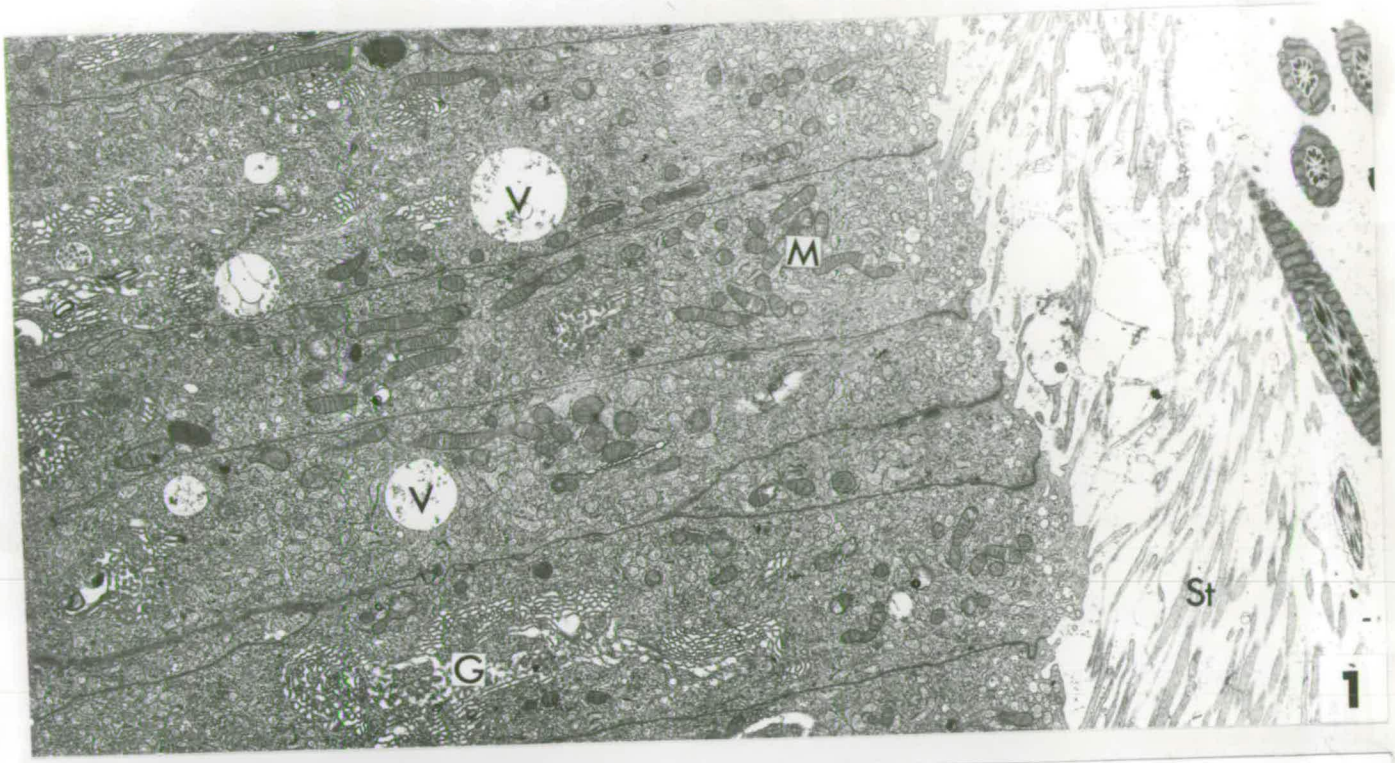
Deeper in the epithelial lining of the epididymis, halo cells are from time to time to be seen (Plate 4, fig. 2). These cells have some resemblance to lymphocytes, but like basal cells they do not have many organelles. They often contain dense lysosomal-like bodies and even if they are not lymphocytes they may perform a similar function. An objection to this interpretation of their function is that they are unable, it would seem, to pass out into the lumen because of the tight junctions between the principal cells.

The basal cells of the epididymis are exceptionally interesting. They rest on the basement mambrane of the duct, and by contrast with the principal cells they have few organelles or cell inclusions (Plate 4, fig. 3). Mostly, basal cells are in contact with principal cells and appear to have a special association with them in that the two interdigitate in a complex manner and exhibit definite adhering junctions at the cell surface[163]. When basal cells are in contact with halo cells these junctions are not to be seen and the plasma membrane surfaces are smooth.

In material obtained from a vasectomized man whose testis and epididymis had been exposed to x-irradiation, we have observed that the basal cells in the head of the epididymis (intermediate part of the middle segment) contained a lot of lipofuchsin pigment granules (Plate 4, fig. 4). In the same material, similar granules were seen in the principal cells and Suzuki[164] believes that these are associated with the absorption of luminal debris and other material that may be

Figure 9.10 (1) Electron micrograph of principal cells of the hamster epididymis. V, Vacuoles; M, Mitochondria; G. Golgi cisternae. x 7,500

(2) Clear cell in the epididymis of the hamster. V, Vacuoles; L, Lysosomel-like bodies; CN, clear cell nucleus; PN, Principal cell nucleus. x 7,500 (Reduced $\frac{1}{2}$ on reproduction)



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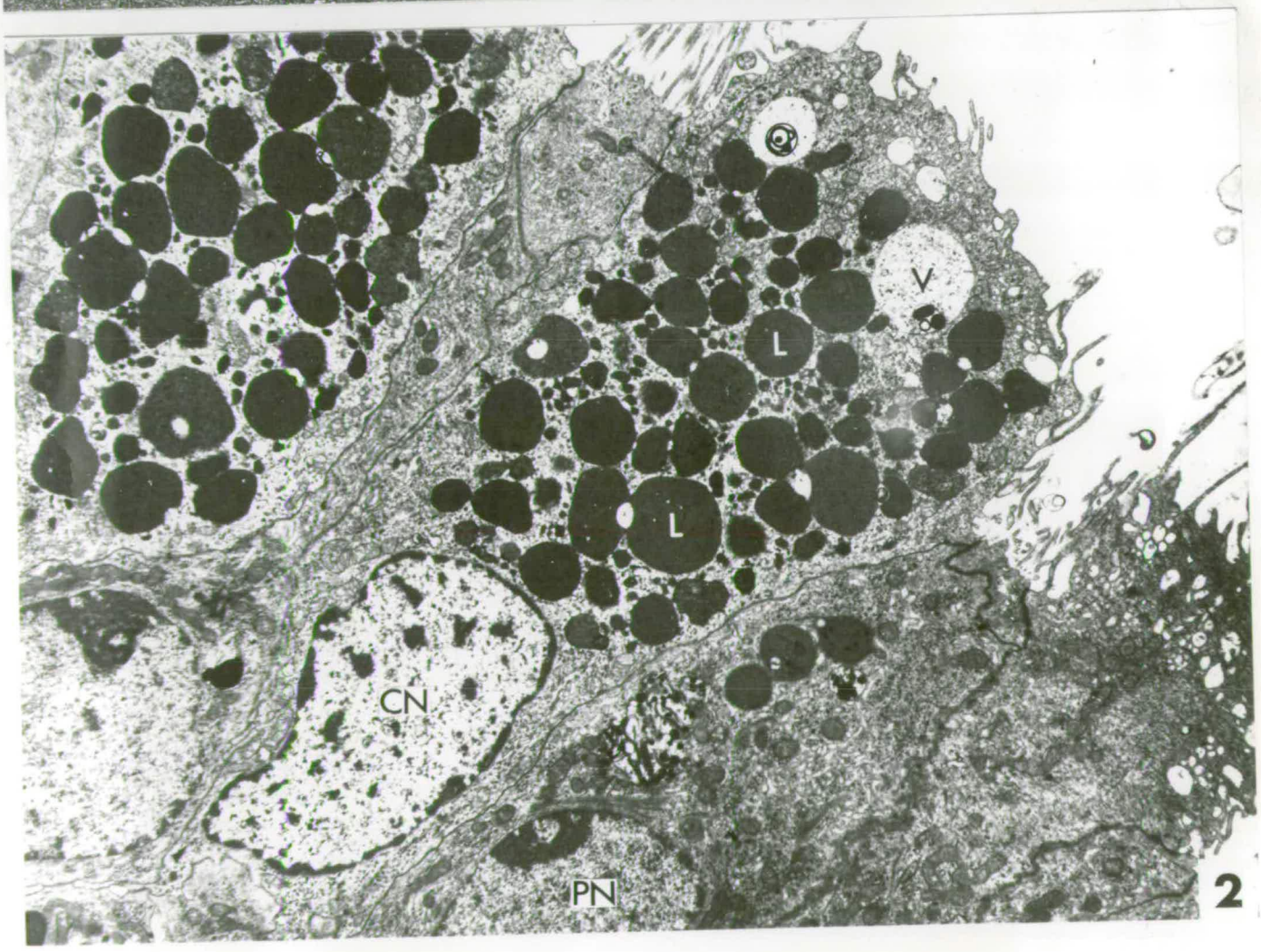

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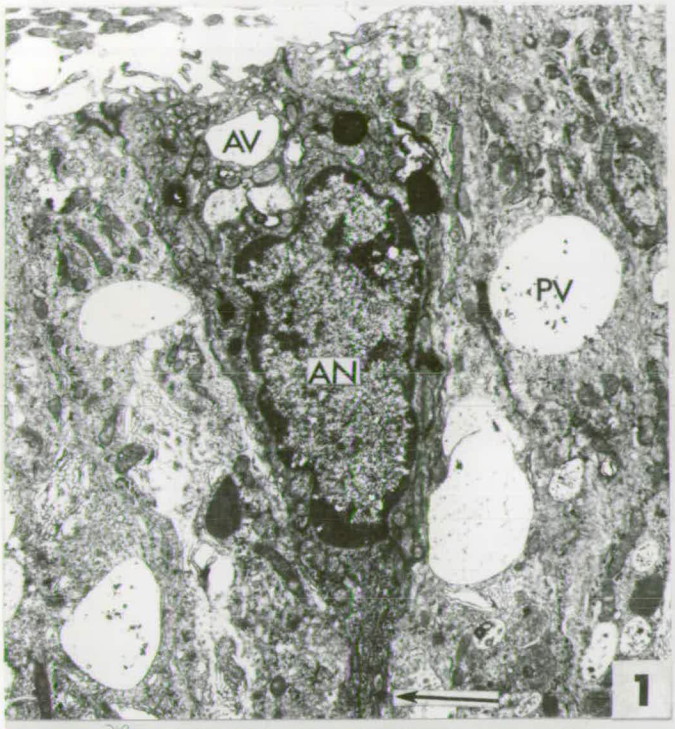
Figure 9.11 (1) Apical cell of hamster epididymis. AV, Apical cell vacuole; AN, Apical cell nucleus; PV, Principal cell nucleus. Arrow pointing to cytoplasmic extension (see text). x 7,500

(2) Halo cell in hamster epididymis in between principal cells. HN, Nucleus x 15,000

(3) Hamster basal cell. P, Rough ER of principal cell; BN, Basal cell nucleus. x 15,000

(4) Human basal cell (x-irradiated). PN, Principal cell nucleus; D, Desmosome, BN Basal cell nucleus; BM, Basement membrane; LP, Lipofuchsin body. x 7,500 (Reduced $\frac{1}{2}$ on reproduction)

(These electron micrographs were prepared by Miss F. Suzuki. Staining with lead citrate and uranyl acetate)


AV
PV
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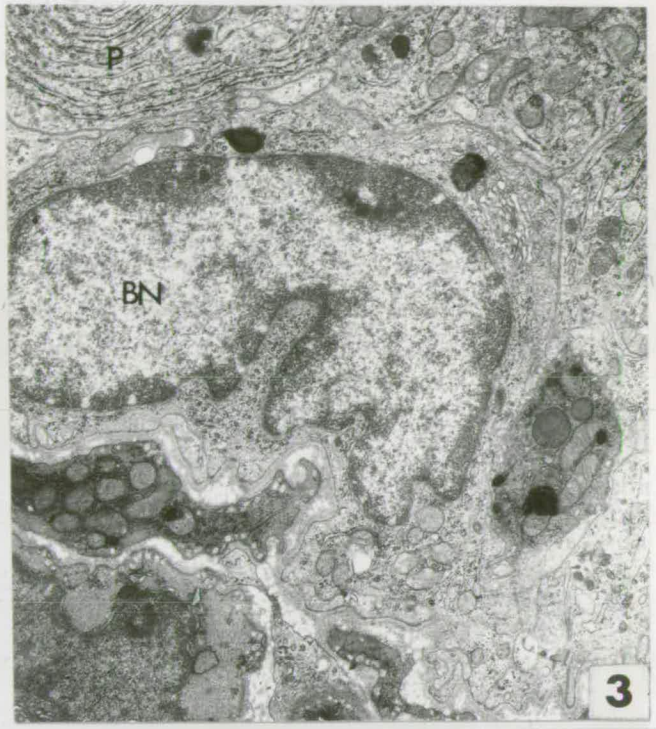
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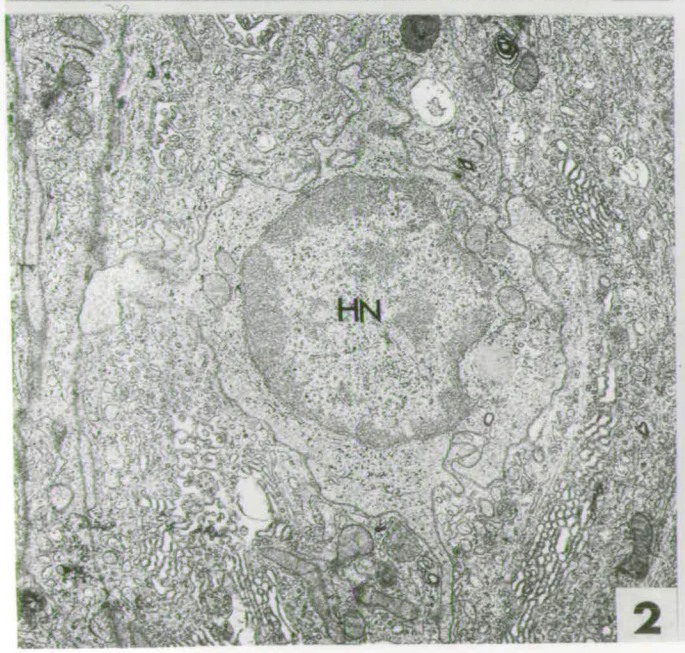
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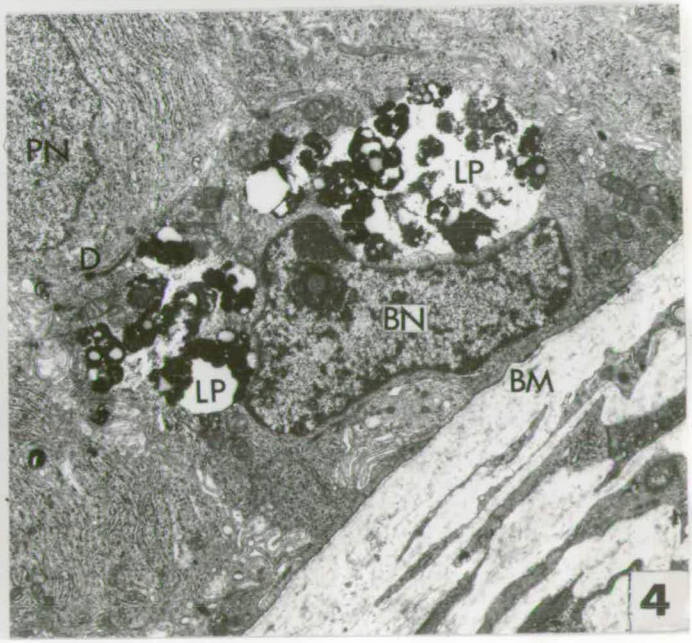
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passed from the principal cells to the basal cells. If this is true, the basal cells may effectively act as scavengers.

Loose areolar tissue surrounds the tubule of the epididymis, binding its convolutions together and containing a number of wandering cells. Plain muscle surrounds the tubule also, as might be expected, and continues as a massive muscular wall in the ductus deferens. Spontaneous contractions in upper regions of the epididymis are more marked and more rapid than those in the tail of the epididymis[165, 166, 135]. This might be expected on the grounds of differences in functional demand between the two areas bearing in mind that only the tail of the epididymis contributes to the emission reflex, but that higher levels of the duct are concerned with sperm transport.

The blood supply to the epididymis has been studied in great detail by Kormano[167, 168] and Kormano & Penttila[169, 170]. These workers demonstrated the distribution of capillaries arising from arteries running in the connective tissue septa between the epididymal convolutions, and they showed that there is more permeability of blood vessels in the head of the epididymis than in other regions. Hamilton[163] believed that this may be related to fenestrations in the capillary wall, but it should be rewarding to examine vascularity and blood flow in a wider variety of species, since there are quite striking species differences in the structure of the epididymis. Detailed information about vascularity, blood flow and capillary permeability are extremely important to an understanding of epididymal physiology, because the facility with which

substances pass from the blood stream to the lumen of the duct is clearly of great significance. Moreover, the manner in which epididymal cells handle materials passed to them is important and has led to an interest in the chemical composition of epididymal plasma, since this must be intimately connected with the problem.

It is important to bear in mind when trying to interpret the chemistry of epididymal fluid that whilst the basic contribution is from the testis, materials are added or withdrawn as the fluid traverses the epididymis. Thus, the chemical composition of the plasma at different levels of the epididymis should be instructive in terms of the activity of epididymal cells, particularly when compared to the composition of testicular plasma. Spermatozoa themselves might affect the composition of the plasma, however, particularly if they degenerate and some intracellular constituents leak out.

A further problem in attempting to study epididymal plasma is that only very small quantities of fluid are available for analysis, that the fluid is difficult to collect without contamination by blood, lymph and epididymal cells, and that resort has often been made to post mortem material. Chemical estimations on post mortem material are bound to be quantitively suspect, but even so, definite patterns in the chemistry of epididymal plasma have been established and appear to be remarkably constant between species. Changes in the concentration of inorganic ions are perhaps outstanding in this context.

Scott, Wales & White[171] showed a progressive decrease in the concentration of sodium and chloride ions from the rete testis to the tail of the epididymis in rams and a comparable effect has been demonstrated in bulls and boars[172, 159, 173, 174] and in rats[175]. The sharpest decline in sodium ions evidently occurs in the head of the epididymis, but, by contrast, potassium increases in concentration in the head of the epididymis and only declines afterwards, except in the rat where it continues to become more concentrated right through to the tail of the epididymis[175]. The concentration of divalent ions such as calcium and magnesium changes little throughout the epididymis and of particular interest is the fact that in spite of substantial changes in sodium, potassium and chloride, the osmotic pressure of epididymal plasma remains relatively constant throughout the length of the duct. Similar values have thus been obtained for the osmotic pressure of rete testis fluid and fluid from the tail of the epididymis respectively[175]. However, there is a discrepancy between the osmotic pressure calculated from total ionic strength and that obtained by direct measurement. The calculation assumes, however, that sodium, potassium, chloride and bicarbonate are the main ions concerned in osmotic regulation and on this assumption the discrepancy is apparently greater with epididymal plasma taken from the tail of the epididymis than with rete testis fluid. It seems, therefore, that other substances must be involved in the maintenance of osmotic pressure in the epididymal fluid, particularly towards the distal

extremity of the duct, but although several substances, including glycerylphosphorylcholine, present themselves as possible candidates for such a role, the true situation is as yet unknown. It is, nevertheless, worthwhile examining organic constituents of the plasma and their possible relationship to sperm maturation and sperm preservation.

Glycerylphosphorylcholine or GPC occurs in progressively higher concentrations in tubular plasma between the testis and the tail of the epididymis[159] but since seminal plasma contains much less GPC, the concentration in ejaculated semen is relatively low[176]. The precise mechanism of GPC synthesis is unknown, but it is accepted that it is produced in the epididymal epithelium and is not the result of breakdown of phospholipids in the spermatozoa[177]. The precursors and enzymes concerned in this synthesis are still unknown, but Martan & Risley[178] attempted to explain GPC synthesis and secretion by proposing a so-called "holocrine secretory cell cycle" involving the clear cells. Unfortunately, this interesting suggestion has not received support in that in some species no clear cells are to be found[179] and in any case, cell turnover would involve replacement, and frequent mitoses would be anticipated. Such mitoses are not evident and indeed the cell population of the epididymis appears to be particularly static. Clearly, more work is needed on the synthesis and function of GPC. It may contribute to the maintenance of the osmolarity of the epididymal plasma, but until its degree of dissociation in the epididymal lumen is known, it is not possible to judge this.

The protein content of tubular fluid also increases between the rete testis and the cauda epididymidis in rams and bulls[174]. Setchell, Hinks, Voglmayr and Scott[180] found that the concentration of glutamic acid followed a similar pattern and Setchell, Scott, Voglmayr & Waites[181] implied that some of this amino acid was synthesized in the epididymal cells.

Hamilton[163] has speculated that this amino acid might be the result of glutamine metabolism and might be derived from the formation of glucosamine-6-phosphate from fructose-6-phosphate. This possibility is suggested by a high level of epididymal sialic acid which represents amino sugars that might be bound to polysaccharide, lipid or protein. Sialic acid is undoubtedly associated in some way with the metabolism of epididymal cells and accordingly appears to be related to the level of androgen, upon which the metabolism of these cells is so dependent[182].

Details of carbohydrate metabolism in the epididymis are not yet available, however, and more experiments in this field should provide valuable information about the metabolism of the cells. A number of relevant enzymes have been identified, but most attention has so far been focused on determining the distribution of these enzymes by histochemical methods and the location of PAS-positive material, particularly that which is resistant to diastase.

As might be expected, species differences exist, but the distribution of glycogen, for instance, seems to be fairly extensive, having been reported in the basal cells of the human epididymis[183] and in the principal cells of certain regions of

the epididymis in other species[184, 185, 186, 187]. Maneely[188] suggested that PAS-positive particles were secretory granules and might represent a source of mucoprotein for assisting the transport of spermatozoa through the epididymis, but Nicander[189] is of the opinion that the particles are associated with absorption.

Fouquet & Guha[190] have demonstrated glycogen synthetase and phosphorylase in the epididymis of the hamster and Allen[191] has shown that in the epididymis of the mouse, glucose-6-phosphatase is variably distributed throughout the length of the duct. The presence of glucose-6-phosphatase is especially interesting in that in other tissues it is concerned with the release of sugar into the blood stream. The question thus arises as to whether this applies in the epididymis or whether the enzyme is associated with the release of carbohydrates into the lumen of the duct. The occurrence of glycosidases, including β-galactosidase and β-N-acetylglucosaminidase, is diffuse within the cells of the epididymis and it seems that the presence of β-glucuronidase is variable[192].

Allen & Slater[193] have studied the lactate dehydrogenase-diphosphopyridine-diaphorase system in the epididymis of mice and whilst the enzymes seem to be homogeneously distributed in the cytoplasm, there is a marked apical concentration of lactate dehydrogenase in the head of the epididymis. Lactate, succinate, and malate dehydrogenases have also been reported in the head of the epididymis in bulls[194] and Niemi[195] has described the presence of succinate and β-hydroxybutyrate dehydrogenases in the apical cytoplasm of the ductus deferens in rats.

Since lactate dehydrogenase and β-hydroxybutrate dehydrogenase are involved in glycolysis and in the oxidation of fatty acids respectively, glucose-6-phosphate dehydrogenase is concerned in pentose cycle activity, and succinate and malate dehydrogenases are vital enzymes in the Kreb's cycle, it is clear that a whole new area of research into the metabolism of epididymal cells is available for exploration. Allen & Slater[196] have also recorded the presence of esterases in the epididymis of the mouse and the activity of nearly all these enzymes is affected by testosterone. The presence of hydroxysteroid dehydrogenases in the epididymal cells[197], therefore, is particularly interesting in view of their association with steroid metabolism. 3-β-hydroxysteroid dehydrogenase, for example, is needed in the conversion of pregnenolone and dehydroepiandrosterone to progesterone and androstenedione respectively, and Moniem[198] has described the distribution of this enzyme in the epididymis of the rat. Taken together with evidence of steroid synthesis by the cells of the epididymis *in vitro*[199, 200, 201], it seems possible that the epididymis may synthesize steroids *in vivo*, but even if it does, the physiological significance of this activity is at present obscure.

Acid phosphatase and β-glucuronidase are localized as discrete granules and may be regarded as being lysosomal[202]. Their respective association with the metabolism of phosphate and mucopolysaccharides suggests that they might be involved in the metabolism of substances absorbed from the lumen of the duct.

Hydrolytic enzymes such as alkaline phosphatase, adenosine triphophatase and adenosine monophosphatase occur in the sub-epithelial connective tissue of the epididymis and in endovascular structures. Judging from their physiological activity in other parts of the body, it is reasonable to assume that these enzymes are involved in active transport between the vascular structures and the epithelial cells lining the duct. The occurrence of alkaline phosphatase activity in the apical parts of epididymal cells similarly suggests active transport between the cells and the contents of the lumen also.

Lipid metabolism in the epididymis is extremely obscure, although the presence of carnitine[203] is interesting and should be investigated further, particularly if it is associated with phospholipids and hence, with cell membranes.

It is difficult to be sure of the origin of all the enzymes and other organic substances occurring in the epididymal plasma and this emphasizes the difficulties of interpreting the chemical composition of the fluid. The enzyme glutamic-oxaloacetic transaminase (GOT) appears to arise mostly from the spermatozoa, especially if they degenerate[204]. It has been suggested that an increase in LDH following castration is related to degenerating spermatozoa[205] and yet, GPC appears to have its origin in the epididymal cells. Interrelationships between the epididymal cells, the epididymal plasma and the spermatozoa are, however, important, and, therefore, the control of metabolic activity in the epididymal cells, and its ultimate influence on the spermatozoa, need to be studied.

3.2.1. Hormones and the Epididymis.

That the epididymis is dependent upon androgen for its metabolic activity is well known and so it is not surprising that the maturation and viability of spermatozoa are also androgen-dependent[206, 207]. Normally, the androgen is testicular in origin, but there is some evidence that pituitary-controlled extra-testicular androgen might also be involved[207].

Niemi & Tuchimaa[208a] have shown radioactively labelled testosterone to be mostly concentrated in the wall of the epididymis but Back[208b] has shown that the concentration of radioactivity in the lumen of the cauda epididymidis is greater than that in the cells lining that area. Thus, the exact mechanism of action of the hormone is as yet unknown, but it seems that, as in several other steroid dependent tissues, it acts by becoming attached to "primary receptors" in the cells[209, 210, 211]. However, the active form of hormone in the epididymal cells is not yet established and although it is known that in a number of androgen dependent tissues, testosterone is reduced to dihydrotestosterone (DHT), there is still no unequivocal evidence that in the epididymis DHT is the active form. But the finding of Lubicz-Nawrocki[212] that DHT is equipotent or even more potent than testosterone in maintaining the viability of epididymal spermatozoa suggests that DHT might well be an intermediate metabolite of testosterone in the cauda epididymidis. This applies even more to 3α, 5α-androstanediol which has been shown to be much more potent than testosterone in maintaining sperm viability in the epididymis[212].

Thus, quite a lot is known already about the hormonal control of epididymal function and recently, considerable advances in this subject have been made. Important gaps still remain, however, and include the passage of hormones from the bloodstream to the cells at different levels of the epididymis and the manner in which epididymal cells utilize androgen. No doubt the biosynthesis of enzymes is governed by androgen[213] so that various secretion products will in their turn be under its control. The synthesis of messenger RNA might also be influenced by androgen[214] and it is interesting that in the epididymis of the golden hamster, Moniem[215] has shown that removal of the testes results in a loss of histochemically detectable RNA from the epididymal cells.

Another interesting role of androgen is to stimulate or at least to regulate contractile activity in the epididymis[216]. Rhythmical contractions of the testes have been observed[217] and these would appear to be in part due to activity of the testicular capsule[218] but also to contraction of the seminiferous tubules themselves[219, 220]. Hormonal control of these latter contractions has not been fully explored although Niemi & Kormano[221] have demonstrated the sensitivity of the contractile elements to oxytocin. These testicular contractions presumably serve to flush out the spermatozoa and fluid into the epididymis via the efferent ductules, but Macmillan[222] showed that the passage of spermatozoa through the epididymis is not dependent upon a vis a tergo from the testis and, therefore, the androgen dependent contractions of the epididymis would appear to be responsible for the transport of spermatozoa through the duct.

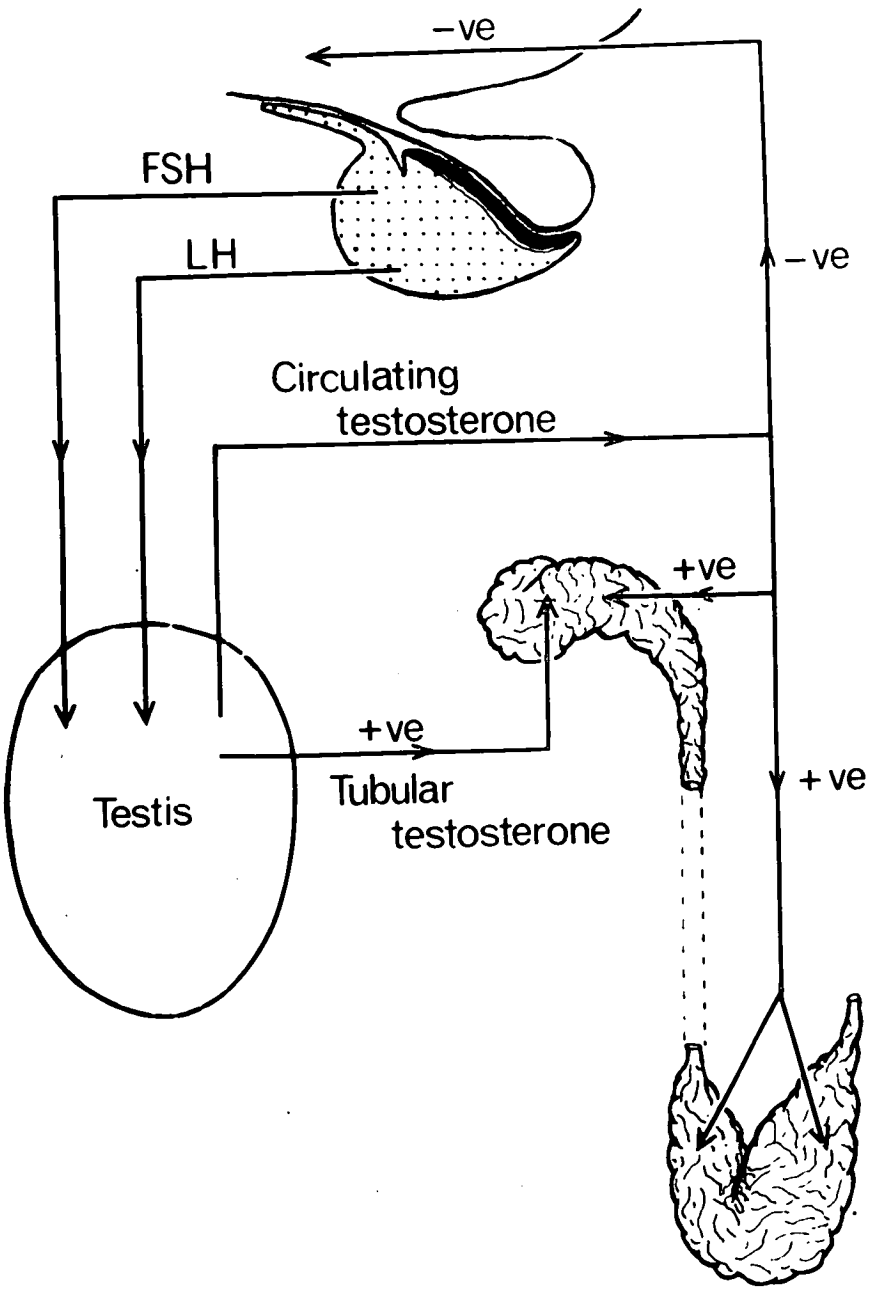


Figure 9.12 Diagram illustrating how the epididymis receives testosterone from two different sources, and the indirect role of the pituitary on epididymal function.

Finally, it has been shown that when androgen is withdrawn by removal of the testes, epididymal spermatozoa not only lose their viability and rapidly degenerate but may completely disappear from the lumen[205, 207]. This also occurs in some seasonally breeding mammals when androgenic levels decline at the end of the mating season[23] and in these mammals it appears to result from autolytic rather than phagocytic activity. This is not to say that phagocytosis such as has been reported in the human epididymis by Phadke[147] may not occur, but it does not seem to be the main means for eliminating epididymal spermatozoa in situ. Available evidence suggests, therefore, that very little dissolution of spermatozoa occurs in the epididymis provided the levels of circulating androgen are adequate. Both species and individual differences may be expected, but the situation poses an interesting question in relation to vasectomy, where presumably hormone levels remain normal.

Although circulating androgen is obviously indispensible to epididymal function, testicular fluid, which is reasonably rich in testosterone, might also participate. It has been shown by Reid[223] that development of the caput epididymidis, unlike that of the cauda epididymidis, is dependent, upon a direct connection with the testis. Moreover, it is known that proximal regions of the epididymis and the efferent ductules are primarily absorptive and it is possible that cells of these upper regions take up testosterone together with other substances. In the cauda epididymidis it is doubtful if the epididymal plasma has

much testosterone in it, although the position might be different in early pubescent animals[224] and no firm data exist in this connection. It seems that whilst the cauda epididymidis is primarily dependent on circulating androgen, the cells of the caput epididymidis might require tubular testosterone in addition (Text - Fig. 8). Tubular testosterone may, of course, be important to the spermatozoa themselves, particularly in the early stages of maturation, but there is as yet no conclusive evidence that androgens act directly on spermatozoa in the epididymis.

4.4. Other Accessory Organs of Reproduction

Secretions of the epididymis undoubtedly contribute to the fluid portion of an ejaculate, but the main volume is made up of secretion from the prostate and bulbo-urethral (Cowper's) glands, and particularly, in those species where it exists, from the seminal vesicle. Fluid, especially pre-coital fluid, is also added by urethral glands, and in stallions, the glands of the ampulla of the ductus deferens contribute most of the 'tail-end' portion of an ejaculate[225]. The anatomy and physiology of these accessory organs differs widely between species and the well known and comprehensive review of Mann[177] is recommended for information on the subject.

Nevertheless, it is worthwhile emphasizing in the present context that these glands are all sensitive to circulating steroid and sometimes they appear to respond to them in rather a special way. It is not surprising, therefore, that in addition to hormonal studies on the epididymis, investigations have been undertaken recently to determine how the cells of the prostate and seminal

vesicles deal with steroid that is delivered to them in the blood stream.

Injected testosterone has been shown to be converted into dihydrotestosterone (DHT) in the prostate, seminal vesicles and preputial glands of the rat[226]. In the prostate this occurs in the cell nuclei and attempts have been made to characterize the binding protein[227, 228]. *In vitro* studies have shown that prostatic nuclear chromatin selectively binds 5α-DHT[229] and whilst testosterone metabolism yields DHT, androstanediol and androsterone, only DHT is retained by the nuclei[229].

In the rat, testosterone is converted more readily to DHT in prostatic cells than in the cells of the seminal vesicles[230] and this sort of information may be most valuable when trying to interpret the response of accessory glands to various hormonal conditions. More detailed information on this fascinating topic will surely be anticipated with interest.

The penis and scrotum are to be included in external accessory organs of reproduction and in different species there are a number of specialized accessory reproductive glands including preputial glands and such secretory structures as the dorsal gland on the back of the rock hyrax. Many of these glands are also sensitive to steroid, but since they constitute such a large subject in themselves they will not be considered here.

THE ANALYSIS OF SEMEN

5.1 General appearance of ejaculates

The analysis of semen is usually undertaken for the purpose of predicting an individual's fertility, but it may also be of

value in diagnosing abnormalities of the male genital tract. It is noticeable, however, that over the last fifteen years, few new methods of semen appraisal that are of immediate value to the clinician have come to light, although well known tests have been modified from time to time. The appraisal of semen in domestic animals has been reviewed in great detail by a number of authors[231], but since it has been recognized in man that the husband is frequently the infertile partner in a childless marriage, and since both AIH and AID have been more widely used in recent years, assessment of semen quality in the human being has become increasingly important. The following remarks will, therefore, be confined mainly to the problem of semen analysis in man.

It is important to be aware at the outset, that the fertility of a man cannot be predicted as accurately as that of many domestic animals. Several semen characteristics in bulls, for instance, have been correlated with fertility[232], but this cannot be achieved in man with the same precision since adequate control data are almost impossible to obtain and since there is much more variability in semen quality even among apparently fertile males. Thus, many assumptions have to be made when using semen quality as an indicator of the fertility of a man, whilst a statement about abnormal function of his male reproductive organs may be made with more certainty.

Functional status of the reproductive organs may be revealed in the general physical characteristics of an ejaculate and this applies to both human and animal semen. Unlike the semen of most animals which is odourless, however, human semen has a characteristic tarry smell which is due to the organic base 'spermine'[177]. The

ejaculate is greyish in colour and contains gelatinous material which liquifies on standing at room temperature. Usually this liquifaction takes about 20 minutes to complete and the significance of the process in semen appraisal will be mentioned later.

Typically, a man ejaculates between 2 and 4 ml of semen, although occasionally much larger volumes are produced. Voluminous ejaculates are to be viewed with some caution though, because they are often associated with a low sperm concentration and even low total sperm numbers. It is not uncommon (personal observation) for complete azoospermia to be associated with a large volume of ejaculate.

Occasionally, of course, there may be contamination of an ejaculate with urine and discolouration of semen samples may arise also from other causes such as infections of the reproductive tract, blood pigments (icteric patients may discharge yellow-coloured semen) and detritus. Sometimes, freshly ejaculated semen appears streaky due to the presence of cellular debris and microscopically, human semen is well known to contain prostatic casts. However, a good quality ejaculate will appear as fairly clear fluid once liquifaction is complete. After this time the motility of the spermatozoa is optimal and after measurement of ejaculate volume, microscopic examination may next be undertaken.

5.2 The Spermatozoa

An early test in the analysis of semen is the test for motility of the spermatozoa. In the semen of bulls or rams, where the sperm concentration is between a million and four million/μl,

motility may be assessed quantitively be measuring impedance change frequency (ICF). This is not possible with relatively dilute samples and so the microscopic observation of sperm motility is still undertaken in the analysis of human semen. Various methods have been described for assessing motility and that of Harvey[233] is quantitively the most useful. However, motility is frequently scored arbitrarily and this is not entirely valueless with an experienced observer, particularly since the assessment usually involves observations on the quality of sperm movement as well as an estimate of the number of motile spermatozoa. MacLeod[234], for instance, uses the term 'motility index' in combining the percentage of motile spermatozoa with the rate of forward progression of the cells.

Nevertheless, there is plenty of evidence from animal experiments that on its own, sperm motility is a poor criterion of potential fertilizing capacity. For example, sperm motility may be retained following fairly low dose x-irradiation, yet the ability to fertilize may be lost. Clinically, therefore, poor motility is more meaningful than good motility, because asthenospermia (defined by Eliasson[235] as less than 50% motile spermatozoa) at least indicates that something is wrong.

That some motile spermatozoa may be infertile is perhaps not surprising, because situations might be envisaged where mid-piece activity is normal, but where the nuclear DNA of the spermatozoa or the lipoglycoprotein complex of the acrosome is adversely affected. It has been shown, for instance, that linkage between

DNA and nuclear proteins may be altered and aberrations in the amino acid composition of the nuclear proteins may occur[236]. Such changes may not necessarily influence motility.

In human semen, optimal motility is only seen when liquifaction of the sample has taken place and, therefore, when there is a failure of liquifaction, the addition of a trypsin-like agent to the semen may be required before proper examination of the spermatozoa can be made.

The whole question of sperm motility has been admirably reviewed by Nelson[237], but although several papers on the subject have appeared since, it is evident that much more research is required before the mechanisms of motility, and therefore, the significance of motility, can be fully understood.

The concentration of spermatozoa in semen only acquires significance when several samples are examined, because any one sperm count may reflect the efficiency of emission and ejaculation rather than sperm production. By examining a number of samples from each patient, however, and by ensuring that a constant period of sexual abstinence is maintained before the samples are produced, a general picture of a patient's sperm production may be obtained.

Counting the spermatozoa in diluted samples using a haemocytometer slide is the usual method of assessing sperm concentration in human semen. Absorptiometric methods would be much quicker and probably more accurate, but their development in this context is hampered by the variable presence of crystals and cellular debris as well as a tendency for various diluents

to cause clumping of the spermatozoa. Nevertheless, more efficient methods are needed and more work in this field is demanded. Similar difficulties are experienced with the use of electronic cell counters[238, 239] although some of the problems can doubtless be overcome by suitable adjustment of the diluents.

A sperm concentration of less than 30,000,000/ml in a human ejaculate is usually taken as constituting oligospermia, but the significance of sperm concentration over total sperm number is not clear. Pregnancies have occurred when the husband's sperm count has been less than 10,000,000/ml and it might be emphasized that if semen volume is large, the total number of spermatozoa in an ejaculate might be considerable. Thus, low sperm counts may not necessarily signify a quantitative deficiency in sperm output but may rather indicate excessive production of seminal plasma. Invariably, however, so-called 'low density' samples are not very potent and when artificial insemination is prescribed for the condition it is necessary to use only the 'high sperm' fraction. This subject will be discussed again later.

Sperm concentration is markedly affected by the frequency of ejaculation, so that when a semen sample is examined, it is important that the period of sexual abstinence before the sample is produced is known and preferably standardized. Without this information a semen analysis is essentially valueless. Frequent ejaculation, it has been claimed, may almost empty the sperm reserve in the tail of the epididymis[240] since the sperm count first declines sharply but later stabilizes at a low level. If this is true, then the phenomenon provides a means of assessing

sperm production as opposed to sperm output, but since the efficiency of emission also decreases with repeated stimulation, interpretation of these effects must be made with care. The subject is an important one, however, and should be examined more closely.

In domestic animals there is an inverse relationship between the fertilizing capacity of an ejaculate and the incidence of certain morphologically abnormal spermatozoa. But in man, the significance of sperm abnormalities is more difficult to ascertain, because a wide variety of morphological forms of spermatozoa appears to be characterstic. These different forms have been described by a number of workers and have fairly recently been reviewed by Freund[241] who believes that some standard classification is needed. This would certainly facilitate easier communication between different workers and might avoid some confusion, but it is difficult to understand how it would assist interpretation. For example, sperm tails coil in very variable fashion, so to classify them all as "coiled tails" might cause confusion and might obscure differences in the origin of the abnormality.

It is interesting, however, that in man many aberrations in sperm structure occur in the head of the cells and are, therefore, presumably mainly of spermatogenic origin, but more attention should be paid both to the origin and the cause of these abnormalities. Increased interest in the cytogenetic aspects of human male fertility is, therefore, encouraging, and experiments where chromosomal activity is related to sperm

morphology are particularly useful (see, for example, Skakkebaek & Beatty[242]). Human spermatozoa differ markedly in size even in one and the same ejaculate, so that microsperms and macrosperms are easily identifiable. Why should this occur especially in man, what is its significance in terms of fertility and what is the origin of the discrepancy? None of these questions has been answered. Moreover, new methods of scanning, counting and measuring the size of cells are now available and yet they do not appear to have been adequately adapted to the examination of spermatozoa. It is to be hoped that methods will be improved in the future, because MacLeod[243] has shown that from the point of view of practical techniques available for routine diagnostic purposes, there has been little advance since the pioneer studies of Moench[244]. However, from the research point of view, the electron microscope has proved a valuable innovation amd much more is now known about the basic structure of the human sperm cell. Very recent techniques such as the binding of colloid to the sperm cell surface[129] are likely to prove useful in understanding further the development of spermatozoa and their reaction to the environment. The histochemical demonstration of glycoprotein binding[245] also has promise and the microscopic observation of proteolytic activity in spermatozoa is also most interesting.[246]

For many years differential staining has been widely used in the appraisal of semen for determining the proportion of so-called "live" spermatozoa. Nigrosin eosin appears to have been the most popular differential stain, largely, one suspects,

because it is relatively easy to use. In bulls, however, the stain has proved useful in assessing fertility[232] and in several other species it has been shown that differential counts on nigrosin eosin smears are reasonably reproduceable[247]. This stain does not appear to have been widely used in the appraisal of human semen, because opinions differ as to its validity with human spermatozoa. There is no doubt that human spermatozoa respond differentially to nigrosin eosin, but it is difficult to give a meaningful interpretation of the results. However, Eliasson & Treichl[248] have described a method for estimating the percentage of living cells by using supra-vital staining and this may prove to be a more useful technique to human seminologists.

The manometric measurement of oxygen uptake by spermatozoa has long been used experimentally as a measure of their activity, but more recently the oxygen consumption of human spermatozoa has been measured polarographically using Clark electrodes[249]. Normally, oxygen uptake by human spermatozoa is very low and Eliasson[250] has shown that this is due to a factor in the seminal plasma that appears to depress oxidative metabolism in the mitochondria. Thus, in human spermatozoa oxygen uptake is increased after washing in Ringer or upon transfer to a salt solution[251, 252].

Anaerobic and aerobic fructolysis may also be used as measures of metabolism in ejaculated spermatozoa, but, like oxygen uptake, these rates express activity of the spermatozoa rather than their fertilizing ability. Although good quality semen samples contain

vigorously motile spermatozoa, therefore, motility in itself may be regarded as a separate entity from the mechanism of fertilization. The mid-piece of a spermatozoon is the centre of the cytochrome system and in addition to the co-enzyme ATP it has a variety of glycolytic and respiratory enzymes. The isozyme of lactate dehydrogenase (LDH_{IV}) is, for example, sperm-specific. The mid-piece is also rich in lipids, particularly plasmalogen and lecithin. Thus, this area of the spermatozoon represents the source of energy for movement and the enzyme adenosinetrophosphatase (ATPase) which occurs in the tail fibrils is likely to be responsible for the transfer of energy from ATP to contractile elements in the flagellum.

By contrast, the instruments of fertilization lie in the acrosome, which contains enzymes such as hyaluronidase, proteinases, esterases and glycosidases which are released for penetration of the envelopes of the egg.

Since much yet needs to be learned about the chemistry of the interaction of spermatozoa and their fluid environment, chemical tests on the semen must at this stage have a particular place in the diagnosis of malfunction in accessory organs rather than in the prediction of fertility, and on occasions, it is important to distinguish between the two.

5.3. The Seminal Plasma

It is now well known that in a variety of mammalian species the accessory sex organs contribute to the composition of ejaculated semen in a characteristic manner. Thus, in man, integrity of function in the seminal vesicle may be assessed by determining

the content of fructose in the ejaculate as well as inositol, prostaglandins and other substances including enzymes. On the other hand, prostatic function may be revealed by the content of citric acid, particular proteins and acid phosphatase. Thus, in prostatitis, for example, the citric acid content of ejaculated semen is invariably reduced. The possible value of various other seminal constituents as tools in the diagnosis of reproductive dysfunction should also be investigated. In this context, GPC, sialic acid, carnitine, acetyl carnitine, glutamic acid and hypotaurine are of interest. Electrophoretic analysis of proteins is also recognized as being of increasing importance in the diagnosis of abnormal reproductive function in the male, particularly in relation to prostatitis[253].

Enzymes in semen may also yield very valuable information about secretory activity in the accessory organs. Infections of the prostate, for example, not only result in decreased concentration of zinc, cholesterol and citric acid in the semen, but also cause a decrease in acid phosphatase activity[254]. By contrast, the LDH fraction of semen is elevated in prostatitis. Seminal fluid has an additional band to the usual five LDH isozymes and this has been called LDH_x. It is an extremely interesting isozyme in that it appears to be associated with the spermatozoa themselves, and might, therefore, be used for estimating enzyme leakage.

The liquifaction of human semen <u>in vitro</u> is a complex enzymic process, which has been discussed in some detail by Mann[177], but it is important that more details of the exact mechanism involved

are determined, because failure of liquifaction is a surprisingly common defect in human ejaculates. The coagulum may be digested with trypsin, however, and it is interesting that Hirschhäuser & Kionke[255] have demonstrated a specific inhibitor for trypsin in human seminal plasma. These workers have suggested that this substance inhibits proteinase on the acrosome of spermatozoa and thus prevents their penetration into cells other than the ovum. This is an unusual idea and bears further consideration.

6. THE STORAGE OF SEMEN AND ARTIFICIAL INSEMINATION.

6.1 Deep freezing spermatozoa

On the face of it, it might seem difficult to separate the subject of sperm preservation from artificial insemination, because stored semen is usually used when insemination is indicated. However, it is important that the success of semen storage can be tested *in vitro* and it is this aspect of the subject that will be mainly dealt with here.

Although it is well known that cells in general are very susceptible to freezing and thawing, the introduction of protective substances has enabled mammalian spermatozoa to be stored at low temperatures for long periods without any adverse effects on their fertilizing capacity or ability to produce viable embryos.

Since the finding of Polge, Smith & Parkes[256] that animal spermatozoa could be protected against the effects of very low temperatures by the addition of glycerol, an enormous amount of work has been carried out on the subject of deep freezing spermatozoa. Many of the earlier papers have been reviewed by Parkes[257] whose account should be consulted for an assessment

of the original literature. The research has meant that for some time now the semen of animals has been successfully stored at low temperatures for long periods and predictably, attention eventually turned to the possibility of storing human spermatozoa by similar means. The literature in this field is now also quite extensive.

The usual technique used for freezing human spermatozoa is essentially the same as that used with other species, although doubtless each laboratory will have its own special modifications. In Liverpool, we use a method based on that given to us by Jackson (personal communication) and the method is described here as a general guide.

The semen diluent is prepared by adding 7.0 ml of egg yolk to 18 ml of citrate/fructose/glycerol buffer. This buffer consists of 3.2g trisodium citrate (Analar), and 2.2g fructose, made up to 100 ml with distilled water. 28 ml glycerol (Analar) are added to this mixture and the pH is adjusted to 7.4 if necessary. The buffer is stored in 18 ml aliquots at - 20°C. Next, the volume of the semen sample is measured after it has been allowed to liquify and the sample is transferred to a 10 ml conical flask. An equal volume of the semen diluent is then measured into a graduated centrifuge tube and is added to the semen at a rate of ten drops every 3 min, and the two fluids are thoroughly mixed between the addition of each drop. 0.8 ml aliquots of the diluted sample are next transferred to 1 ml ampoules which are afterwards sealed and incubated in a

refrigerator at 5°C for 30 min. The ampoules are then clipped into the top of a cane and allowed to stand in liquid nitrogen vapour in a sealed vacuum flask for 30 min. Finally, the ampoules are quickly transferred to the bottom of the cane and the cane is placed into the liquid nitrogen refrigerator. Each of these steps is illustrated in Plate 4.

The semen is thawed by rapid removal of the ampoule from liquid nitrogen and immersion in water at 35°C for 2 min. It is then usually left for a further 5 min. at room temperature before insemination.

Semen was at first frozen by means of solid carbon dioxide after slow cooling, and samples were taken down to - 79°C, although Bunge[258] has suggested that human semen stores better at - 85°C. Sherman[259] however, described the use of liquid nitrogen for low temperature preservation of spermatozoa and the use of this technique has now become common practice for long term storage. This is not to say that other substances such as isopropyl alcohol[260] which might cool to - 70°C, might not be feasible for short periods of storage.

The history and development of research on frozen human semen has been surveyed by Sherman[261], who explained some of the technical improvements in storage methods that were introduced about 10 years ago, and this worker recommended that further experiments be undertaken to improve diluents and protective substances and to refine the methods of cooling that were then in use. Since then, work in this field has developed considerably, but no substance appears to have superseded glycerol as a practical

Figure 9.13 (a) The preparation of diluent by addition of egg yolk to the buffer

(b) Dropwise addition of diluent to semen sample

(c) Aliquots of diluted semen being placed in ampoules

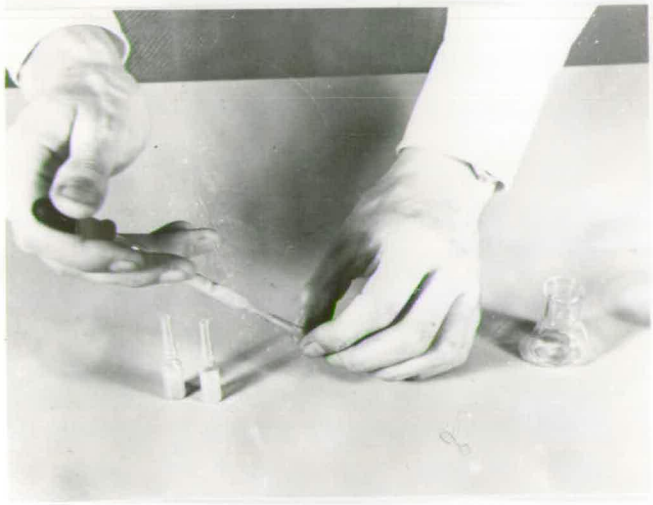
(d) Sealing of ampoule containing sample, care being taken to keep ampoule as near upright as possible so as to avoid heating the mixture

(e) Vacuum flask containing cane with samples in it representing when closed, the second stage of cooling, after an initial stage in a refrigerator at + 5.0°C. About 2 inches of liquid N_2 is placed in the bottom of the vacuum flask.

(f) The last stage of cooling and storage. The cane is quickly transferred to liquid N_2 in an immersion storage tank (N_2 refrigerator (see text)

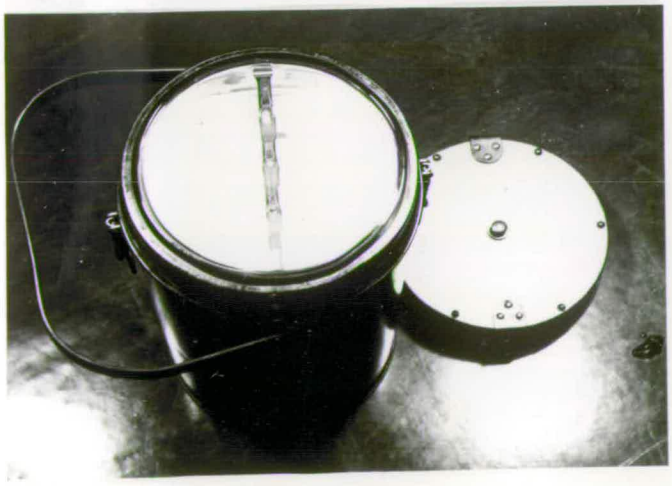
a

b

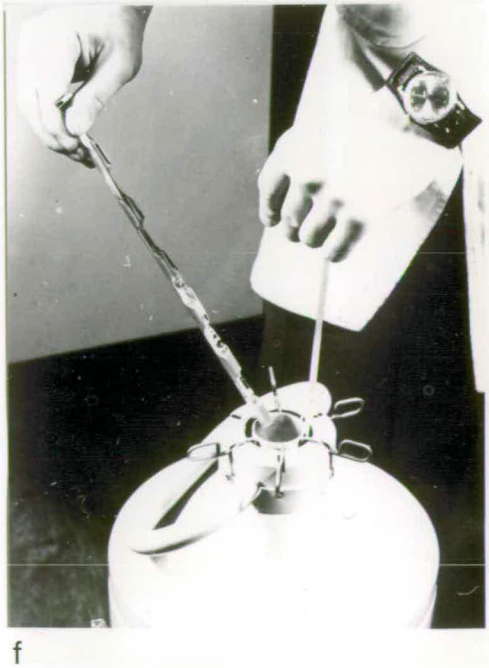


c

d



e



f

protective agent. Richardson & Sadlier[262] showed that the toxicity to spermatozoa of glycerol, dimethysulphoxide, ethylene, glycol, methyl formamide and methyl acetamide, was about the same, but that dimethylformamide and dimethyl acetamide were more toxic when used in the same concentrations, namely 2.5% to 10%. Dimethyl sulphoxide has been widely applied for preserving cells against the effects of freezing and it has been used in the freezing of animal semen[263] but Zimmerman, Maude & Moldawer[264] found that with human semen, glycerol produced better results as far as post-thaw motility and the percentage of live spermatozoa were concerned.

Although actively motile spermatozoa are not necessarily fertile, a good semen sample contains a large proportion of them. It is thus important in freezing semen to achieve good post-thaw motility and the criterion is widely used as a test for the success of freezing. Differential staining has also been used as a test, (Zimmerman et al.[264]), but it often proves unreliable with thawed spermatozoa (personal observations). There is absolutely no evidence from in vitro studies[265] that sperm DNA is adversely affected by current methods of freezing and a very large number of normal babies have been born from artificial insemination with semen previously stored at low temperatures. An abnormal baby resulting from the use of stored semen may, therefore, be regarded as a statistical fortuity and to reflect either an abnormality of the original semen sample or some defect in the mother. Nevertheless, with AID, it is obviously prudent to choose donors whose ability to produce normal offspring is already proven.

Freezing does cause damage to some spermatozoa, however, so that the percentage of motile spermatozoa is usually reduced and the fertilizing ability of samples may also be affected[266]. Several inseminations are almost always necessary to achieve a pregnancy with stored semen, but it is difficult at present to assess how far this is due to the adverse effects of freezing and thawing, since the same situation often seems to apply when fresh semen is inseminated[267, 268]. The ability of thawed human spermatozoa to penetrate cervical mucus is also progressively impaired by periods of freezing[269].

Attention might well be turned, therefore, to the possible injury caused by freezing to metabolic activity in the spermatozoa, to the function of the acrosome and to the sperm surface. Ackermann[270] produced evidence that exposure to low temperatures may influence sperm hyaluronidase activity, which is normally fairly constant, and he held the view that glycerol preserves this activity in a cold environment. Healey[271] showed also that the ultrastructure of spermatozoa in some domestic animals was damaged by freezing. The spermatozoa of chinchillas and rams were cited as examples, and it was claimed, that freezing caused gross deformity in the acrosome of boar spermatozoa.

A further problem is that there are species differences in the ability of spermatozoa to withstand the effects of freezing. It has been shown, for example, that in different species of primates survival of spermatozoa after freezing varies considerably[272]. So with the complicated genetic history of different human beings, variation in the resistance of human spermatozoa

to freezing is predictable and has been borne out in the light of experience. Thus, the semen of different men responds variably to low temperature storage and unfortunately different ejaculates from one and the same person sometimes vary in their resistance to freezing.

Undeniably, therefore, this whole subject needs looking at much more closely with particular attention being paid to the control of media and to the entire question of interactions between spermatozoa, diluents and temperature. Freund & Wiederman[260] made an approach to this subject and Norman, Goldberg, Porterfield & Johnson[273] studied the prolonged survival of human spermatozoa in chemically defined media. This sort of work needs to be expanded and a thorough study of cryobiology in spermatozoa, particularly human spermatozoa, is sorely needed, since work in this field has, for a variety of reasons, tended to be piecemeal. Obviously, if the demand for artificial insemination in man is to become more widespread, safe and efficient methods of semen storage must be ensured.

The indications for artificial insemination in man, the ethics of AID and the actual techniques employed are not the province of the biologist, since they form the background to clinical decisions. But looking into the picture from the outside as it were, the potential value of artificial insemination in certain conditions is clearly apparent. The greatest call for AID is likely to be in cases of unresolved azoospermia in the husband, but rhesus incompatibility and predicted disease arising from recessive genes are also situations where AI may be indicated.

Requests for semen storage for purposes of AIH are on the increase, since vasectomy has become more popular as a means of birth control. Opinions differ as to the desirability of storing the semen of patients before vasectomy and it seems that it is not generally encouraged, although again this is not for the biologist to decide. The storage of semen before vasectomy is certainly quite feasible on technical grounds.

There may be indications for storing human semen from patients who have been subjected to unilateral orchiectomy for seminoma, when the contralateral testis appears to function normally, but where it might have to be sacrificed later in the course of treatment.

Finally, there is the question of concentrating successive ejaculates in cases of oligospermia. Available data offer little guide as to the value of attempting artificial insemination with these cases, except that it must surely be a last resort and the portents are that it will mostly yield disappointing results. The plight of an infertile couple may demand that AIH with bulked semen by undertaken, but it would seem commonsense to ensure beforehand that the couple have been trying to reproduce naturally for a period of several years.

The indications are that the high sperm fraction should always be used with artificial insemination, particularly when ejaculates of large volume and low sperm density are used[274]. A method of obtaining such partitioned ejaculates has been described by Harvey & Jackson[275] and by Harvey[276]. Generally, the first portion of an ejaculate to be discharged is the high sperm fraction,

although in some 6% of cases this sequence is reversed[274].

The whole subject of artificial insemination in man has been fully reviewed by Behrman[277, 278] and these reviews should be consulted for an overall interpretation of the subject.

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PRELUDE

The papers appearing under this heading concern work which was carried out under supervision and are not intended as part of the main body of the thesis. They are only included in order to provide a background and illustrate the experiments that led up to the present work.

VII

SOME EFFECTS OF SCROTAL INSULATION ON THE SEMEN OF RAMS

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The potential ability of semen to impregnate the female is dependent upon the morphological and physiological conditions of the spermatozoa and probably also, on the character of the accessory secretions. All these depend in turn upon the normal functioning of the male organs of reproduction, and any deviation from the normal may therefore affect first the semen characteristics and ultimately fertility.

Definite lesions observed in the testes or accessory glands *post mortem* or after castration may indicate the aetiology of some cases of infertility, but if maintenance of the entire animal is to be considered first, neither slaughter nor gonadectomy is of much value for diagnostic purposes. Testicular biopsies can be used for diagnosis of spermatogenic activity, but the results may be misleading. Diagnosis based on the examination of semen offers however, a useful approach, and it was thought that by a systematic study of seminal characteristics during the periods of induction and recovery from artificially produced sterility, much might be learned which would be of value for the diagnosis of male sterility in general.

Scrotal insulation, which is known to induce sterility in rams, without having any detrimental systemic effect on the animal, and the effects of which are reversible, was chosen as the simplest and most easily controlled experimental method. Rams were used in the studies since their pendulous testes provide good material for scrotal insulation.

The primary interest in this work, therefore, has not been the fertilizing capacity of the semen, but the use which can be made of the semen picture as an indicator of the activity of the male reproductive apparatus.

MATERIALS AND METHODS

The experimental rams were subjected to insulation of the scrotum by the application of rubberized cloth packs lined with kapok and suspended from the back by a webbing harness (Figure 1). Semen collections were made by means of an artificial vagina (Walton, 1945). The semen was examined for semen volume, sperm density, visual

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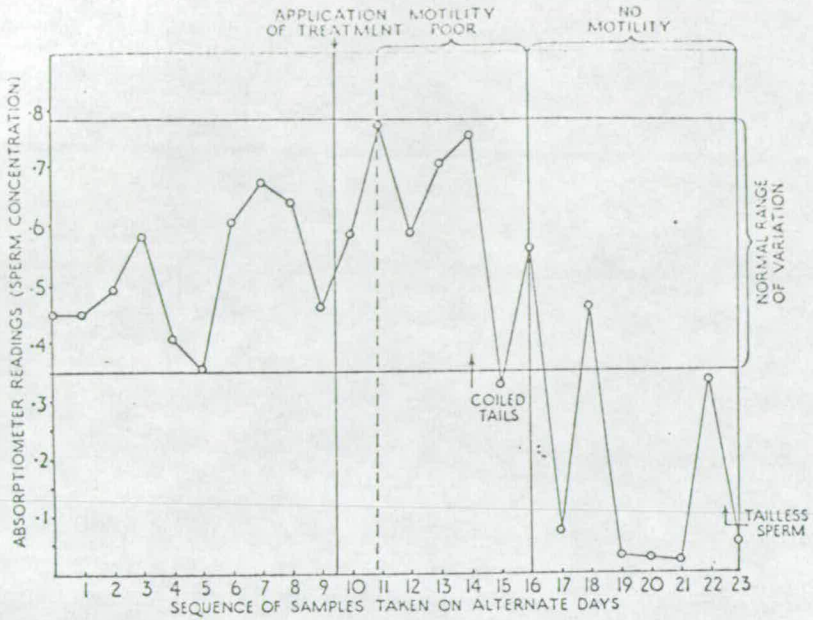
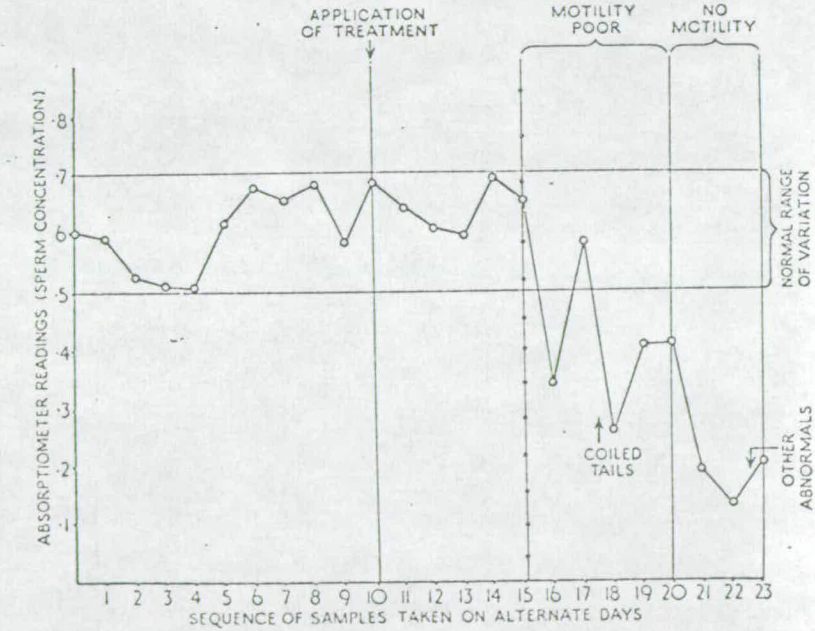


FIGURES 5 and 6

Changes in concentration of spermatozoa in two rams due to insulation of the scrotum.



motility, sperm morphology and percentage of dead spermatozoa. Motility was also assessed by measurements of electrical impedance-change-frequency (Rothschild, 1949) and metabolic activity measured manometrically. Fructose determinations were carried out on ejaculates in the hope that they would provide an indicator of male-sex hormone activity (Mann and Parsons, 1947). The percentage of live and dead spermatozoa was estimated by means of the nigrosin-eosin differential stain (Hancock, 1951). The same slides were also used in assessing morphological changes in the spermatozoa, and any observed changes were verified from time to time by phase-contrast microscopy on whole semen. The density of spermatozoa was measured absorptiometrically (Bishop, Campbell, Hancock and Walton, 1954).

EXPERIMENTAL DESIGN AND RESULTS

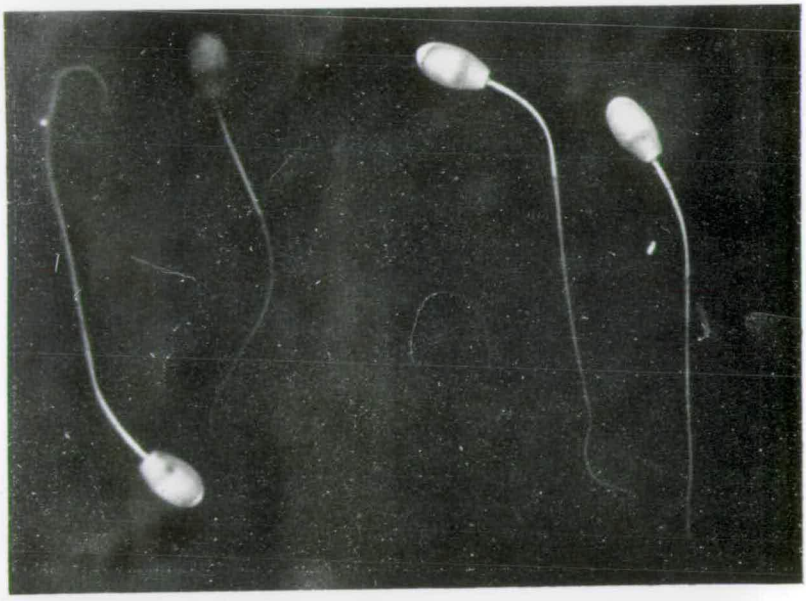
Experiment 1. This experiment was carried out to investigate the effect of continuous scrotal insulation on ram semen as revealed by the tests described above. Six rams were used, of which three were controls. Semen collections were made on approximately alternate days. After a number of trials to establish the normal semen quality of each ram, three experimental animals chosen at random had packs applied to their scrota. When definite symptoms of azoospermia appeared in the semen of these animals, the packs were removed and the process of recovery and regeneration followed.

The results of this experiment showed that as oligospermia increased, so did the percentage of dead spermatozoa and the percentage of morphologically abnormal spermatozoa. The morphological changes were characterized by the appearance in the semen of a high percentage of spermatozoa with coiled tails, and which were mostly motile and non-eosinophilic (Figures 2-4). This phase was followed by a marked fall in the sperm concentration of semen samples, which at the same time contained almost all headless eosinophilic spermatozoa. Two examples of these changes are shown in Figures 5 and 6. As would be expected, at this time, the impedance-change-frequency (referred to hereafter as I.C.F.) also diminished, as did the total O_2 uptake of the samples. The fructose concentration, however, was elevated (Figure 7). These changes were reversed in not quite the same manner during regeneration.

It has been noted during this experiment that there was considerable variation in individual response to the treatment, as shown, for example, with I.C.F. measurements (Figure 8). This represents the actual responses of three rams and illustrates the fact that the ram semen which was most severely damaged by the treatment was that which began to degenerate first and regenerate last. The reverse was the case with the ram semen which withstood the treatment best.

FIGURE 1

A Suffolk and a Romney Marsh ram with scrotal packs applied showing the method of insulating the scrotum.



FIGURES 2-4

Photomicrographs showing stages in the degeneration of ram semen due to insulation of the scrotum.

FIG. 2. Normal ram spermatozoa.

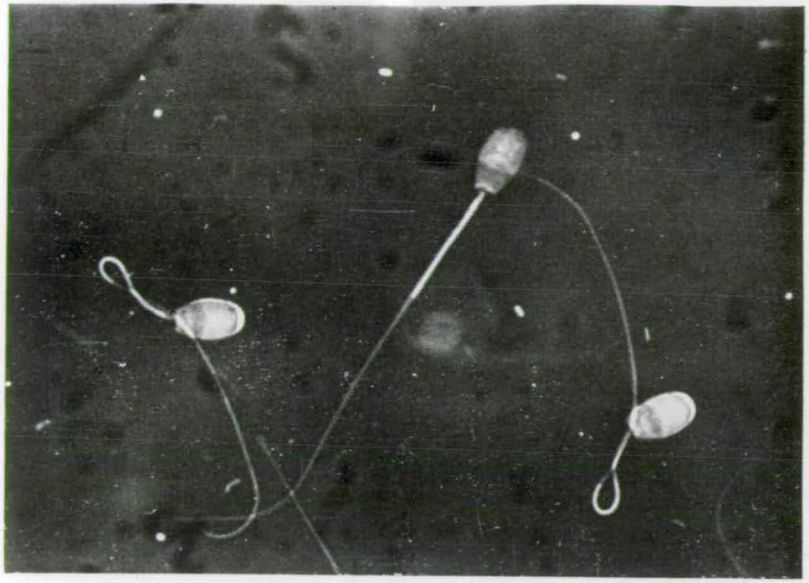


FIG. 3. Coiled tailed spermatozoa.

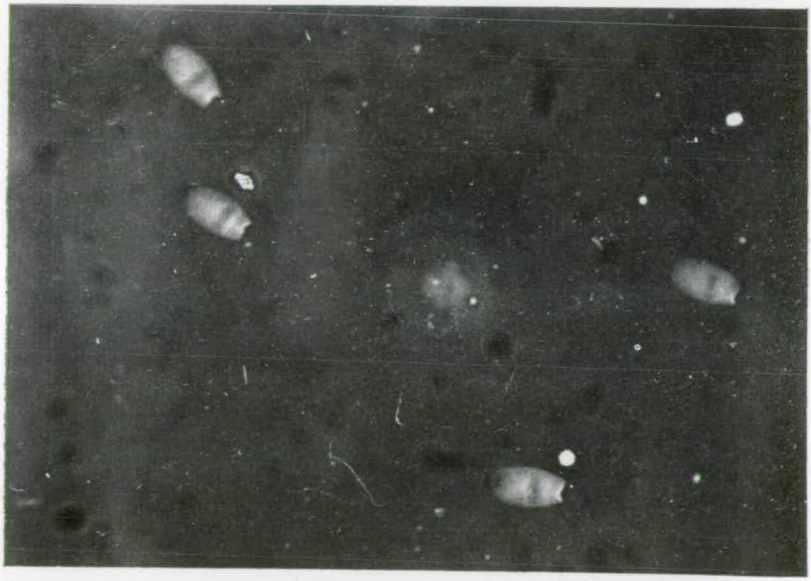


FIG. 4. Decapitated spermatozoa.
All these sperms are stained with aqueous nigrosin-eosin (×1300).

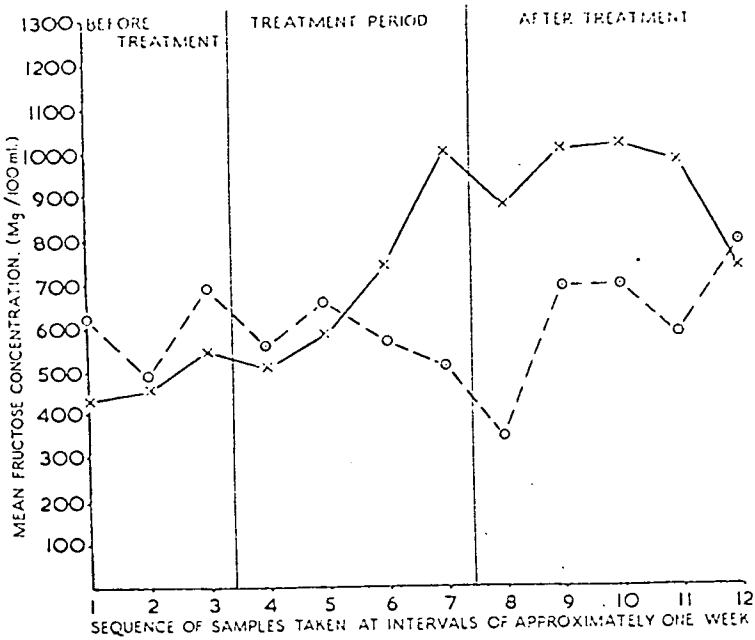


FIGURE 7

The effect of scrotal insulation on the fructose concentration in semen. The mean fructose concentration of treated animals (× ——— ×) is shown to rise during scrotal insulation compared with the controls (○ - - - - ○).

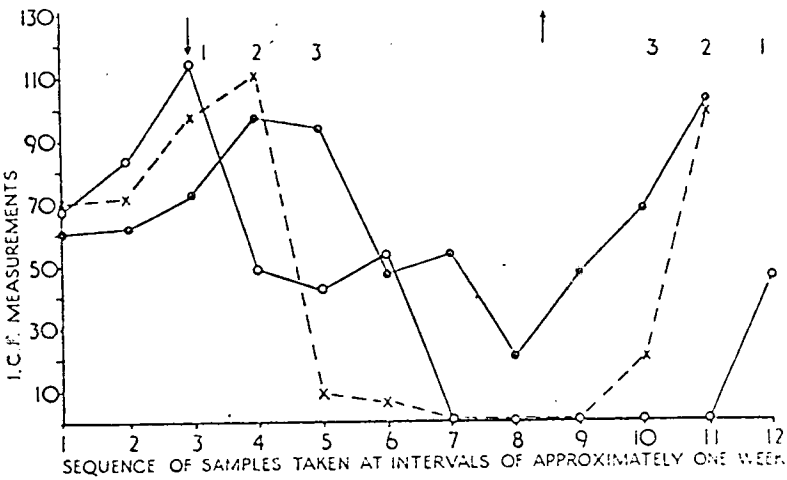


FIGURE 8

The effect of scrotal insulation on impedance-change-frequency of spermatozoa from the semen of three rams. The vertical arrows represent the point of application and then removal of the scrotal packs. The order in which degeneration started and regeneration occurred may be compared, and the relative degree of degeneration noted.

A decrease in the total O_2 uptake of semen samples from the treated animals was to be expected as the number of living spermatozoa in the ejaculates decreased. There was some indication, however, that I.C.F. began to decline before significant changes in sperm concentration or morphology developed. It has not yet been statistically calculated whether the O_2 uptake per sperm cell was initially affected by the treatment.

The content of fructose began to rise after scrotal insulation, concurrently with the fall in sperm density. This change might have indicated an increase in testosterone activity-level, but no increase in libido was observed. During regeneration, however, the fructose levels slowly subsided and the animals did appear to lose their libido to some extent.

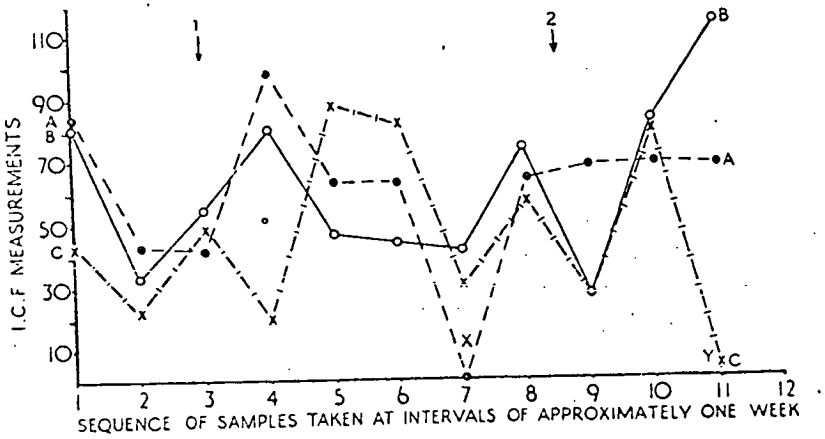


FIGURE 9

Impedance-change-frequency measurements, illustrating the transitory change brought about by 24 hours of scrotal insulation and the delay in effect.

A. I.C.F. of spermatozoa from a ram treated at point 1.

B. A control untreated ram.

C. Ram treated at point 2.

At points X and Y a high percentage of decapitated spermatozoa appeared in the semen.

It must be emphasized, however, that at this time (March and April) the end of the normal breeding season of sheep in this country was approaching. A more complete analysis of this material will be published elsewhere (Glover, 1956).

During regeneration, the morphological picture appeared normal before the sperm density returned to its usual level, but a detailed study of the material is not yet complete. A high percentage of 'knobbed' spermatozoa (Hancock, 1949) was, however, a characteristic feature of the recovery phase.

Experiment 2. Phillips and McKenzie (1934) found that seminal changes occurred in rams after 7-8 days of scrotal insulation, and Gunn, Sanders and Granger (1942) showed that at least 5 days of such treatment were required. The present experiment was performed to determine whether the delay in appearance of abnormal semen was due to a accumulative effect of temperature on the testes, a belated reaction of spermatozoa already in the cauda epididymidis, or to the time taken for affected spermatozoa to pass through the epididymis and vas deferens. Six rams were scrotally insulated as before but for a much shorter period. After 24 hours' application only, the packs were removed and regular semen examinations subsequently made. The results on these six rams showed that only 24 hours of scrotal insulation brought about some seminal change (Figure 9). The change was characterized by the sudden but transitory appearance of decapitated spermatozoa in the ejaculates which did not occur for some time after treatment (Table I).

In each of the six rams from which semen was collected every alternate day, headless spermatozoa appeared after periods varying from 17-24 days following treatment; the percentage of such spermatozoa in the ejaculates varied from 15.0%-67.5%. During the periods between treatment and the appearance of these spermatozoa, no significant changes were apparent in the semen. In only one case was there a significant fall in sperm density (Ram 489). The peak in the degree of decapitation in samples was rapidly attained and was only transitory, so that the normal semen picture was soon resumed.

TABLE I

Ram number	*Time before effect (days)*	*Maximum decapitation before effect* (%)	*Maximum decapitation caused* (%)	*Mean sperm density before effect* ($\times 10^6$/*ml.*)	*Mean sperm density during effect* ($\times 10^6$/*ml.*)
489	19	7.00	69.0	3.97	2.37
492	24	2.00	61.0	3.92	3.72
170	22	3.50	8.0	4.45	4.24
264	17	9.00	25.50	3.75	3.77
236	17	7.50	23.75	3.65	4.01
214	19	1.00	15.50	3.86	3.95

Experiment 3. The previous experiment suggested that the delay in the appearance of decapitated spermatozoa in the semen was primarily due to sperm migration. The third experiment was therefore planned to determine whether the rate of emptying the *cauda epididymidis* by ejaculation influenced the time of onset of seminal changes. Four rams

were used in the experiment; two were collected from only once a week and two were collected from three times daily for 57 consecutive days. As in the previous experiment, the semen was examined for morphological changes.

This experiment showed that though seminal degeneration began to develop slightly sooner in the frequently ejaculating rams than in the infrequent group, the difference was too small to be attributed specifically to the ejaculation frequency. Contrary to expectation, in the regenerating testes, recovery was slightly slower in the frequently ejaculating group. The ultimate difference in time of recovery was very small, but the results might have been affected by the approaching end of the breeding season.

DISCUSSION

Crew (1922) suggested that probably the aspermatic condition of the undescended testicle was due to the fact that this organ required a lower environmental temperature for its optimum functioning than that of the abdomen. Abundant support for this belief has been provided by subsequent experiments in which the testicular temperature has been elevated through artificial cryptorchidism or scrotal insulation. Lagerlöf (1934) showed that insulating the scrotum of bulls resulted in certain abnormalities of the spermatozoa and eventually in azoospermia. In the present experiments similar results have been obtained with scrotal insulation of rams. In addition, however, using some of the more recent methods of semen appraisal, a number of new observations have been made.

Previous work on the effects of scrotal insulation in large animals has been performed with prolonged application of the treatment and immediate effects were not detected, but Asdell and Salisbury (1941) demonstrated disturbances of spermatogenesis in the rabbit after 24 hours of experimental cryptorchidism. Moore (1951) showed that damage to the testis of guinea pigs occurred after only 10 minutes application of heat (6° C. above scrotal temperature) to the scrotum. Experiments described herein have indicated that in rams only 24 hours of scrotal insulation is necessary to produce seminal effects.

Great care is needed in the interpretation of results obtained from experimentally induced seminal changes. It is important to recognize that changes in semen characteristics may not all take place simultaneously and furthermore, that values obtained for one characteristic may be affected by other different characteristics. In other words, there is considerable interaction between the results of various tests; for instance a positive correlation between a decrease in I.C.F. and sperm density occurring during degeneration may be primarily due to the simultaneous increase in the proportion of dead and abnormal spermatozoa. Such a

close correlation does not occur during recovery, since the content of dead and abnormal cells is less, although a low sperm density occasioned by gradual recuperation exists during this period. A similar effect does not necessarily occur in cases of infertility which are met in practice where conditions are not controlled. The precise timing at which changes develop, may be one of the most important contributions to the study of artificially induced seminal degeneration.

The nigrosin-eosin differential stain has shown that the spermatozoa with coiled tails were mostly non-eosinophilic and they were seen to be motile; this indicates that spermatozoa may retain normal permeability and be metabolically active even though abnormal morphological change is apparent. The question remains, however, whether some metabolic change may not have occurred in such cells. From the tests applied it would seem that metabolic changes in spermatozoa which are morphologically normal, occurred in these experiments, as revealed by a fall in I.C.F. prior to change in live density, or the appearance of abnormal forms of spermatozoa in the ejaculates. The possibility that temperature elevation may affect spermatozoa already in the epididymis, despite their normality on morphological grounds, is being further investigated by similar methods.

Following insulation of the rams' scrota, a slight but significant increase in seminal fructose has been noted, which only slowly declines to its normal level. The possible significance of this finding will be discussed elsewhere (Glover, 1956).

In all these experiments there was variation between rams in the time of onset of seminal degeneration irrespective of whether the treatment was applied for a short or prolonged period; but in all cases some time elapsed after the treatment began before gross morphological change appeared in the semen. This delay it was considered, was due either to the time taken for the spermatozoa to migrate down the epididymis or to a delayed heat effect on testicular or epididymal sperm. If the former explanations are correct, and testis function is affected immediately on application of the scrotal insulation, accessory gland changes, such as a rise in fructose concentration, might be expected in the semen at once, since migration would not be involved here. However, in the experiments, the fructose concentration rose concurrently with the sperm changes. Meschaks (1955) using neutral steroids to indicate male-sex hormone activity, found likewise that increased excretion of these substances after scrotal insulation was detectable at the same time as abnormal sperm appeared in the semen. These findings seem to indicate that symptoms of testicular dysfunction, including endocrine effects, after scrotal insulation are delayed. As regards fructose, the concentration might be increased within the seminal vesicle before the change became apparent in the semen. On the present evi-

dence, therefore, the simultaneous appearance in the semen of cellular and accessory gland changes may be coincidental and any explanation must be conjectural.

The delay in appearance of seminal changes following a short period of scrotal insulation, however, strongly indicates that damage occurs either in the testis itself or high up in the epididymis rather than in the cauda (Glover, 1955), because in this case no accumulative heat effect on spermatozoa in the cauda epididymidis would be possible. This delay might provide a guide to the migration time of the spermatozoa down the epididymis, but only if the decapitation effect occurs immediately after treatment, and whether this occurs or not remains unknown. Such a method of assessing migration was suggested by McKenzie and Berliner (1936). These workers used prolonged insulation of the ram's scrotum, however, and this might itself have influenced the rate of migration, and provided the possibility of an accumulative heat effect. Such an influence could not be present with the short period of treatment. It is distinctly possible, however, that the cause of semen changes due to short periods of scrotal insulation may differ from those due to more prolonged treatment. A further possibility that decapitation of spermatozoa in either case is a fairly immediate effect cannot be recognized by histological methods at present at our disposal, and therefore may not be considered analogous to changes in the histology of the testis.

The experiment indicates that the sperm-forming mechanism of the ram is more susceptible to interference with the normal thermo-regulation than has been previously demonstrated.

It is interesting to note that in experiment 2, the rams which normally had excessive amounts of wool on the scrotum (Romneys) were less susceptible to scrotal insulation than were those with scrota normally devoid of wool (Suffolks). The reverse was the case, however, in experiment 1 (long period of treatment). This would seem to warrant further study.

In the last experiment it was demonstrated that the frequency of ejaculation had little effect on the time of appearance of degenerative changes in the semen. From this it can be concluded that the rate at which the epididymis is emptied of spermatozoa on scrotal insulation is virtually the same in relative sexual repose as in frequent ejaculation. This might be due to masturbation or to increased resorption of spermatozoa from the epididymis or vas deferens. These possible explanations are supported by the observation that oligospermia did take place in the infrequently ejaculating animals, and that the frequency of ejaculation did not markedly affect recovery.

SUMMARY

(1) Three experiments involving scrotal insulation of rams have been described.

(2) The effect of prolonged scrotal insulation on the semen of three rams has been demonstrated by the use of several tests, including estimates of semen volume, sperm density, sperm morphology and the percentage of dead spermatozoa. Motility of spermatozoa has been assessed by visual examination under the microscope, and quantitatively by measurements of electrical impedance-change-frequency.

(3) The effect of only 24 hours of scrotal insulation on the semen of six rams has been described.

(4) It has been shown that during scrotal insulation, frequency of ejaculation has little influence on the time of appearance of abnormal spermatozoa in the semen.

ACKNOWLEDGMENTS

This work was carried out with the aid of a Research Scholarship and Wellcome Fellowship of the Animal Health Trust, and was directed by Dr. A. Walton; I am most grateful to him and to Dr. T. Mann, F.R.S., for help and guidance. I am indebted to Mr. A. V. Guntrip of the Animal Research Station, Cambridge for taking the photographs and photomicrographs.

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[*From the Proceedings of the Physiological Society*, 25–26 February 1955.]
Journal of Physiology, **128**, 22*P*.

The effect of a short period of scrotal insulation on the semen of the ram.

By T. D. Glover. *A.R.C. Unit of Reproductive Physiology and Biochemistry, Cambridge*

Characteristic seminal degeneration in rams produced as a result of scrotal insulation was first shown by Phillips & McKenzie (1934). Others, like Gunn, Sanders & Granger (1942), found that these changes were not apparent in the semen unless the scrotum had been insulated for at least 5 days.

In the present work experiments on six rams involving a 5° C elevation of testicular temperature by scrotal insulation for a single 24 hr period resulted in the sudden appearance in the semen of tailless spermatozoa 17–24 days after treatment.

The delay in the onset of seminal changes would suggest that damage to the spermatozoa occurs either during spermatogenesis or sperm migration rather than in the cauda epididymis. Only one ram out of the six, however, showed a significant oligospermia, and thus it would seem that severe inhibition of spermatogenesis does not always occur with acute scrotal insulation.

Variation in susceptibility of individuals to this treatment has been noted. Romney Marsh rams which normally had abundant wool on the scrotum appeared more resistant than the Suffolk rams whose scrota were devoid of wool.

The experiments suggest that the testis may be more susceptible to interference with its normal thermo-regulation than has been hitherto believed.

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J. Endocrin. (1956) 13, 235–242

THE EFFECT OF SCROTAL INSULATION AND THE INFLUENCE OF THE BREEDING SEASON UPON FRUCTOSE CONCENTRATION IN THE SEMEN OF THE RAM

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(*Received* 6 *August* 1955)

SUMMARY

Six rams have been subjected to insulation of the scrotum as a means of increasing the temperature of the testes, and the levels of fructose in semen estimated before, during and after such treatment. It was shown that during the period of scrotal insulation a fairly rapid increase in seminal fructose occurs, which only gradually returns to its normal level after termination of the treatment.

No pronounced effect of scrotal insulation on libido has been established.

Observations on ram semen collected towards the end of the breeding season show that a decrease in seminal fructose occurs immediately before the decline in libido becomes apparent, and at a time when the normal density range of spermatozoa is still maintained.

It is well known that the activity of the testes of most mammals is influenced by temperature, and the function of the scrotum as a thermo-regulator for the testes can be regarded as well established. Interference with the thermo-regulation of the testes, therefore, whether by insulation of the scrotum or by experimental cryptorchidism, results in deficient spermatogenesis. On the other hand, Bouin & Ancel [1903] expressed the view that in bilateral cryptorchidism the accessory sex glands continued to function normally. This is in agreement with observations that secondary sexual characters are on the whole unaffected by cryptorchidism or scrotal insulation, and that the Leydig cells of the testes are not destroyed under these conditions. Moore & Gallagher [1930] reported that, in guinea pigs, 8 months of artificial cryptorchidism produced no apparent effect on the secretion of testicular hormone, but Moore [1951] demonstrated a complete inhibition of spermatogenesis in the same species after only 10 min application of heat to the scrotum. Nelson [1937] used the cytology of the accessory glands as an indicator of the output of testicular hormone, but was unable, by this method, to detect changes in the seminal vesicles of rats before 240 days of experimental cryptorchidism had elapsed. Moore [1944] performed similar experiments on rats using the weight of the seminal vesicle to assess the activity of the male sex hormone. He found that confinement of the testes in the abdomen reduced hormonal activity at first, but if the abdominal position of the testes was maintained for longer periods varying from 20 to 80 days, the hormone

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level remained constant. Apparently, therefore, temperature exerts a more pronounced influence on spermatogenesis than on the endocrine activity of the testes, though the precise effect on the latter requires further study.

It has been shown by Mann & Parsons [1947, 1950] that the production of fructose in the male accessory organs is linked with the activity of testicular hormone and that an increase in the content of fructose in semen can be evoked in response to testosterone. The same authors have also shown that the disappearance of fructose which follows castration can be prevented by injecting testosterone. It has been suggested that the estimation of the initial fructose content of semen could be used as an index of the activity of the male sex hormone [Mann & Parsons, 1947; Mann, Davies & Humphrey, 1949].

During experiments in this laboratory concerned with scrotal insulation in rams, it was noted that occasionally, but by no means regularly, a stimulation of libido occurred in the treated animals. It was therefore decided to repeat these experiments and to show, at the same time, the effect of scrotal insulation on the concentration of fructose in semen. In addition, in order to determine the production of fructose under natural conditions of hormonal decline, the concentration of fructose in ram semen was studied at the end of the breeding season when libido declines.

MATERIALS AND METHODS

Three Suffolk rams and three Romney Marsh rams, housed under the same conditions of nutrition and environment at the Animal Research Station, Cambridge, were used for the experiments. Semen was collected by means of an artificial vagina [Walton, 1945]. The initial fructose content of the semen was determined as soon as possible after collection, because if ram semen is left at room temperature, even for a short time, there is a rapid decrease in fructose owing to a high rate of fructolysis [Mann, 1948]. For a period of 3 weeks prior to any experimental treatment estimations of seminal fructose were carried out on all six rams at approximately 6-day intervals; after this, three rams, selected at random, were subjected to scrotal insulation, the other three being used as controls. The methods used for insulating the scrotum of the rams and for examining the cellular components of the semen under conditions of scrotal insulation are described elsewhere [Glover, 1955]. The volume of each ejaculate was measured and estimations of fructose in the ejaculated semen were then continued until maximum testicular damage was revealed by the overall semen picture in the experimentally treated rams. At this point, the scrotal packs were removed to allow recovery of the testes. When spermatogenesis appeared to have recovered in the treated rams, fructose estimations on the semen of all six animals were continued until spring, i.e. until the libido has diminished or disappeared.

An indication of the intensity of libido was obtained by noting the time that elapsed from the release of the ram, at a point about 3 yards away from the 'teaser' animal, to the moment of ejaculation; this method was based on that described by Chang [1942]. As a rule, rams were used as 'teaser' animals, but in one case (recorded in Table 4) a ewe in oestrus was used instead. The introduction of such a ewe is often necessary at the beginning of the breeding season, because libido of the experimental rams is not fully developed at this time, nor have the animals become accustomed to the procedure of collection.

The fructose content of the semen was determined by the method of Mann [1948], based on the Seliwanoff reaction. The procedure finally adopted for the small quantities of semen available (the volume of ejaculates varying from 0·2–1·5 ml.) was as follows: 0·1 ml. of the semen was diluted with 1·9 ml. of distilled water and immediately deproteinized with zinc hydroxide by adding 2·0 ml. of 2 % zinc sulphate and 2·0 ml. of 0·4 % sodium hydroxide. The sample was then heated in boiling water for 1 or 2 min in order to complete deproteinization, and after shaking, the contents poured through a coarse filter (Green 275); 2·0 ml. of clear filtrate were then treated with 2·0 ml. of 0·1 % alcoholic resorcinol (Analar) and 6·0 ml. of 30·0 % hydrochloric acid. The sample was next heated in a water-bath for 10 min at 85° C, withdrawn, allowed to cool, and the intensity of colour determined in the Hilger absorptiometer using the blue-green filter, number 603.

RESULTS

The effect of scrotal insulation on the level of seminal fructose

The effect of scrotal insulation on the formation of spermatozoa in the ram has been described by several workers, but information concerning the activity of the accessory glands in these circumstances appears to be limited. The results from the present study, however, show that an increase occurs in the concentration of fructose (mg/100 ml.) in semen both during and after a period of scrotal insulation (Table 1). This increase did not appear in untreated control animals. Fig. 1 shows that, in all samples of semen used throughout the experiment, there is an inverse relationship between the concentration or density of spermatozoa and the concentration of fructose expressed in mg/100 ml. of semen. Since variation in the volume of the ejaculates was very small, it may be argued that any increase in the concentration of seminal fructose in the animals subjected to scrotal insulation might have been entirely due to a corresponding decrease in the number of spermatozoa. It therefore became necessary to determine the concentration of fructose in the seminal plasma of each ejaculate. The volume taken up by seminal plasma and by spermatozoa in each ejaculate was calculated from the volume of the ejaculate by determining the sperm number and assuming that each sperm cell occupies a volume of $6{\cdot}5 \times 10^{-8}$ μl. [Mann, 1952, personal communication].

The values for the mean concentrations of fructose in the seminal plasma of both the control animals and the scrotally insulated rams (Fig. 2) shows that there is a definite increase in the level of fructose as a result of scrotal insulation. Since there is little or no fructose in the spermatozoa, the fructose content of whole semen must be due to that in the seminal plasma. In other words, the absolute content of fructose per ejaculate is that of the seminal plasma.

The values expressing the content of fructose per ejaculate (Table 2) were therefore used for analysis. They were analysed in a two-way classification analysis of variance, the two classifications being by weeks and by rams. Each classification was subdivided, the weeks according to whether the measurement was taken before, during or after the period of scrotal insulation, and the ram classification according to whether the animal was in the control or treated group. In the resulting analysis of variance the 'main effects' were for treatment, rams within treatments, periods, and weeks within periods; there were also interactions between these factors.

The variance ratio tests showed that the treatment × weeks, rams (within treatments) × periods and rams × weeks interactions did not differ significantly, but that the treatments × periods interaction was larger than the pooled value of these interactions at the 5 % level of significance. The rams (within treatments) effect exceeded this pooled interaction at the 1 % level of significance. The mean squares used in these tests are given in Table 3. These results mean that, although there were large differences between rams, the difference between the treatment and control groups

Table 1. *Effect of scrotal insulation and the end of the breeding season on the level of fructose in ram semen*

	Fructose concentration (mg/100 ml. semen)			Mean values for fructose concentrations (mg/100 ml. semen)	Mean sperm density (× 10^9/ml. semen)
	Ram no. 1	Ram no. 2	Ram no. 3		
	Animals subjected to scrotal insulation				
Before treatment	280	680	310	423	3·66
	285	720	380	462	2·86
	300	750	580	543	3·82
During treatment	285	620	615	507	4·94
	545	480	685	570	4·96
	620	830	775	742	3·30
	1100	1150	750	1000	2·96
After treatment	700	1130	750	860	2·24
	830	1160	1010	1000	1·68
	1030	1080	945	1018	1·54
	1000	900	1025	975	1·44
	660	500	1035	732	3·56
	360	390	420	383	4·26
Last weeks of the breeding season	410	R	520	465	3·20
	360	R	820	590	3·74
	150	R	760	455	5·00
	280	R	480	380	4·95
	195	R	420	307	5·10
	Ram no. 4	Ram no. 5	Ram no. 6		
	Control animals				
Before treatment	700	530	630	620	2·98
	560	510	425	498	3·36
	840	545	690	692	3·30
During treatment	755	355	545	552	3·36
	845	435	680	653	3·26
	660	635	375	557	4·94
	370	510	670	517	4·66
After treatment	255	445	420	340	4·28
	790	530	760	693	3·90
	640	575	875	697	4·14
	550	820	380	583	3·14
	790	720	875	795	2·86
Last weeks of the breeding season	350	495	590	512	3·38
	510	R	900	705	2·84
	460	R	500	480	5·60
	540	R	R	540	4·75
	400	R	R	400	5·15
	325	R	R	325	5·30

R = ram refused to ejaculate. These cases have been discounted when calculating mean values.

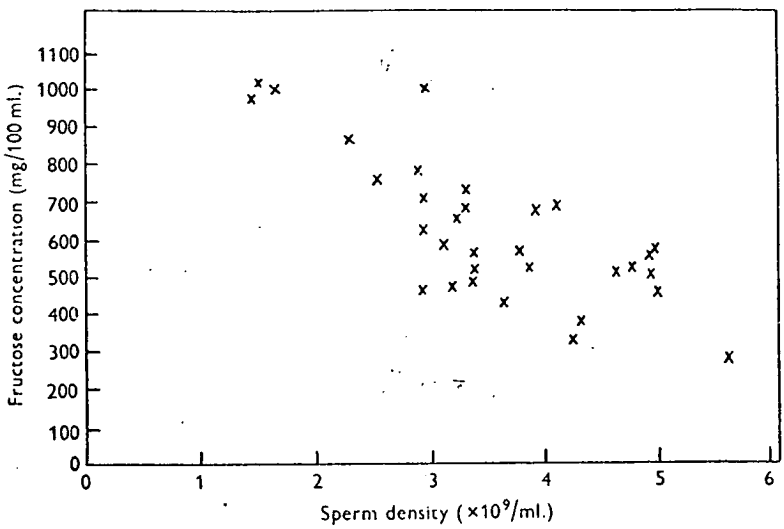

Fig. 1. Scatter diagram illustrating the inverse relationship between sperm density (× 10^9 spermatozoa/ml.) and fructose concentration (mg/100 ml.) in ram semen.

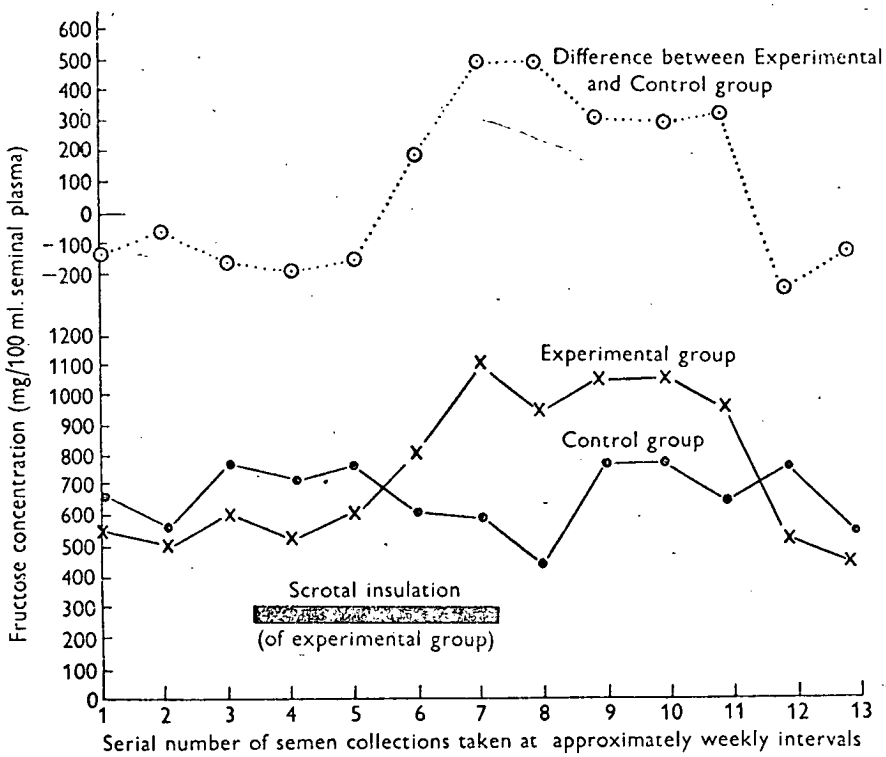

Fig. 2. The effect of scrotal insulation on the concentration of fructose in ram seminal plasma; these results show that the rise in fructose concentration of whole semen (compare Table 1), following scrotal insulation, was not entirely due to changes in sperm density (see Text).

varied significantly from period to period; the mean fructose contents (mg/ejaculate), which are given in Table 1, show that this interaction was due to an increase in concentration for the treated animals initiated during the second (treatment) period. The concentration of fructose was not, however, restored to its normal level immediately after the scrotal packs were removed; the change was only gradual, and even 3 weeks after the end of treatment the treated animals were producing a higher concentration of fructose in the semen than the controls.

Table 2. *Effect of scrotal insulation and the end of the breeding season on the content of fructose in the ejaculates (=fructose content of seminal plasma)*

	Animals subjected to scrotal insulation				Control animals			
	Fructose content (mg/ejaculate)			Mean values for fructose content (mg/ejaculate)	Fructose content (mg/ejaculate)			Mean values for fructose content (mg/ejaculate)
	Ram no. 1	Ram no. 2	Ram no. 3		Ram no. 4	Ram no. 5	Ram no. 6	
Before treatment	2·80	2·80	3·62	3·07	2·10	4·24	11·60	5·98
	3·50	9·70	5·01	6·07	5·60	5·10	4·80	5·17
	2·55	2·40	5·80	3·58	6·30	4·63	5·91	5·61
During treatment	2·80	8·00	7·38	6·06	3·40	6·94	3·54	4·63
	4·90	5·80	6·85	5·85	4·20	3·05	7·19	4·81
	4·34	6·50	7·75	6·20	4·00	6·99	3·00	4·66
	8·25	8·60	7·50	8·12	3·30	3·83	6·36	4·50
After treatment	4·20	6·90	9·30	6·80	2·10	3·78	2·90	2·93
	6·22	10·20	6·56	7·66	7·50	4·77	4·90	5·70
	6·69	8·30	6·82	7·24	4·60	5·75	8·00	6·12
	5·50	4·50	6·66	5·55	4·40	6·97	4·56	5·31
	3·30	4·50	8·27	5·36	5·10	5·40	4·38	4·96
	2·88	1·75	6·93	3·85	2·40	3·21	5·90	3·84
Last weeks of the breeding season	2·66	R	7·02	4·84	2·80	R	7·65	5·21
	2·52	R	6·55	4·53	1·60	R	6·31	3·95
	2·42	R	5·70	4·01	1·62	R	R	1·62
	1·96	R	4·80	3·38	2·00	R	R	2·00
	0·97	R	4·62	2·79	1·40	R	R	1·40

R = ram refused to ejaculate. These cases have been discounted when calculating mean values.

Table 3. *Analysis of mean squares of fructose content*

Source	Degrees of freedom	Mean square	
Treatment (T)	1	14·50	
Rams within treatments (R)	4	12·68	
Periods (P)	2	2·63	
Weeks within periods (W)	10	4·50	
Interaction: T × P	2	14·97	
T × W	10	3·40	Pooled value = 3·41
R × P	8	1·67	
R × W	40	3·76	
Total degrees of freedom	77		

The concentration of fructose at the end of the breeding season and its relation to libido

It may be seen from Table 1 that, towards the end of the breeding season, the concentration of fructose in the semen tended to decline just prior to the cessation of libido, whereas the density of spermatozoa was not so affected. On the other hand,

the rams subjected to scrotal insulation showed no well-defined increase in libido, in spite of an elevated level of fructose in their semen. However, as can be seen from Table 4, slight fluctuations in libido, as assessed by the time between release of the ram and ejaculation, are difficult to recognize quantitatively owing to very considerable variation within as well as between the rams.

Table 4. *Libido of rams assessed by measuring the time interval (in sec) from the moment of release to ejaculation*

	Animals subjected to scrotal insulation				Control animals			
	Time interval (sec)			Mean value of time interval (sec) for the three rams	Time interval (sec)			Mean value of time interval (sec) for the three rams
	Ram no. 1	Ram no. 2	Ram no. 3		Ram no. 4	Ram no. 5	Ram no. 6	
Before treatment	25·0	25·0	*	25·00	8·0	65·0	20·0	31·00
	77·5	36·0	9·0	40·83	7·0	12·0	9·0	9·30
	39·0	56·0	21·0	33·66	5·0	12·0	205·0	74·00
During treatment	40·1	35·0	39·0	38·03	10·0	12·5	23·0	15·16
	53·0	46·5	41·0	46·83	15·0	15·5	25·0	18·50
	46·8	28·0	24·0	32·93	4·0	18·0	60·0	27·30
	81·0	37·2	18·0	47·73	20·0	12·7	51·0	27·90
After treatment	54·0	57·0	17·5	42·83	6·0	26·0	30·0	24·00
	71·0	39·0	25·5	45·16	4·0	28·8	32·5	28·43
	85·2	41·5	14·0	46·90	8·0	16·5	25·0	16·50
	68·0	44·0	22·0	44·66	5·0	14·0	40·2	21·73
	97·5	103·5	10·8	70·60	9·1	60·5	59·0	42·86
Last weeks of the breeding season	105·2	260·0	21·0	128·73	22·5	125·0	37·5	61·66
	58·6	R	21·5	40·00	16·0	R	215·5	115·75
	90·0	R	46·0	68·00	8·0	R	63·0	35·50
	100·5	R	32·2	66·35	20·1	R	R	20·10
	210·2	R	50·1	130·15	10·5	R	R	10·50
	200·5	R	77·5	139·00	18·5	R	R	18·50

R = ram refused to ejaculate. These cases have been discounted when calculating mean values.

* In this case a ewe in oestrus was used as a 'teaser', whereas on all other occasions a ram was used for the purpose.

DISCUSSION

It is known that the sexual drive of the male is associated with the activity of male sex hormone, and therefore when an increase in libido occurs, a higher level of activity of male sex hormone might be expected. Studies on the effect of elevation of testicular temperature on the activity of male sex hormone have, however, produced conflicting results. Meschaks [1953] reported that after scrotal insulation there was an improvement in the readiness of a number of bulls to serve, particularly in those individuals which previously had a poor libido, and furthermore found that, in bulls, an increase in the excretion of neutral steroids in the urine followed scrotal insulation. This author suggested that these results were probably due to an increase in testicular and adrenocorticotrophic hormone activity. In contrast, Pfeiffer [1937] found a reduction in the size of the seminal vesicles in the rat after exposure to a temperature of 39–40° C in hot cabins, which would suggest a decrease in the activity of the male sex hormone. In the guinea-pig, Elfving [1950] observed a similar effect after the local application of heat to the testes, whereas a variable effect on libido was the result

obtained by Casady, Myers & Legates [1953] from their studies of the effect of environmental temperatures on the testes of bulls. However, the effects of local and general elevation of temperature on the testes are hardly comparable, since a rise in body temperature, such as would result from treatment in hot cabins, presumably produces metabolic changes in other organs which may indirectly influence the testicular function.

In the present experiments it has been shown that scrotal insulation in rams causes a significant increase in the concentration of seminal fructose, and from the work of Mann & Parsons [1947, 1950] it would appear that this could be attributed to an increased level of male sex hormone in the body of the animal. On the other hand, libido did not appear to change significantly after scrotal insulation. This may be due to the fact that the quantitative method which has been adopted for assessing libido was inadequate, or that the level of activity of testosterone after scrotal insulation was still below the threshold value for producing a distinct behavioural response, as expressed by a definite increase in the sexual drive.

The suggestion that the concentration of fructose in semen may be related to the activity of male sex hormone is supported by the observations which show that the levels of fructose begin to decline in ram semen just prior to the cessation of libido which occurs at the end of the breeding season, while the density levels of spermatozoa still remain high. It would thus seem that, under the conditions which were used in the present study, an inverse relationship exists between the spermatogenic and endocrine activity of the testes.

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NIGROSIN EOSIN AS A STAIN FOR DIFFERENTIATING LIVE AND DEAD SPERMATOZOA

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(With One Text-figure)

The purpose of this paper is to describe experiments on the usefulness and accuracy of nigrosin eosin as a differential stain when measuring the proportion of living and dead spermatozoa in samples of ram, boar or bull semen. The importance of this proportion has been shown in the studies of sperm metabolism and morphology which have followed the development of artificial insemination (Bishop, Campbell, Hancock & Walton, 1954) and the nigrosin-eosin stain, in which the dead spermatozoa take up eosin whilst the nigrosin acts as a background, and several other stains which can be used in measuring this proportion, have been widely tested. Easley, Easley & McKenzie (1942) used opal-blue and eosin to stain ram spermatozoa. Mayer, Squiers, Bogart & Oloufa (1951) used this stain as well as fast-green and eosin with human, ram, bull, rabbit, stallion and boar spermatozoa; they found, however, that neither of these methods gave consistently satisfactory results with boar spermatozoa. Blom (1950) and Hancock (1951) described a nigrosin-eosin mixture for use with bull spermatozoa, and Hancock (1953) suggested that this mixture could be used for studying the effects of cold shock on the spermatozoa of the boar as well as those of the bull.

Campbell, Hancock & Rothschild (1953) determined the accuracy of counts obtained when the nigrosin-eosin stain was used with bull spermatozoa, and the original object of the present investigation was to extend their work to the ram and the boar. During the experiments with ram and boar semen, however, certain findings differed from those obtained with bull semen (Campbell *et al.*, 1953) and we therefore include this species in the present study.

MATERIALS, METHODS & RESULTS

Preparation of the smears

Semen was collected by means of an artificial vagina (Walton, 1927, 1945; Glover, 1955), and held at 30° C. The stain used consisted of:

Nigrosin (Gurr)	10 g.
Eosin Y (Gurr)	1·67 g.
Distilled water	100 ml.

except in one experiment when citrate buffer solution was used as the solvent (sodium citrate $[Na_3C_6H_5O_7 \cdot 2H_2O]$ at 32 g./litre, 10 % citric acid to bring the pH of the buffer solution to 6·8). The method of preparing the smears was based on method 2 described by Bishop *et al.* (1954). The staining solution, in a small test tube, was maintained at 30° C, and one drop of semen was added by means of a warmed pasteur pipette. After five minutes one drop of semen-stain mixture was withdrawn and smeared on a slide at 30° C; the smears were mounted in D.P.X. The amount of staining solution used was chosen so that the distribution of spermatozoa on the smear was neither so dense nor so sparse as to make counting impossible; 35–40 drops of the solution were needed for 1 drop of ram semen, but 6 drops were sufficient with boar semen and 8 with bull.

Experimental design and counting technique

In what follows a semen-stain mixture will be referred to as a subsample. Several subsamples were made from each sample of semen, to make possible a measure of the variability between different parts of the same sample—this stage of the preparation technique has been found to introduce

considerable sampling variations in the proportion of dead spermatozoa (Campbell *et al.*, 1953). Two smears were made from each subsample, and counts of the numbers of living and dead cells in two or three groups of 100 spermatozoa were made on each smear. In most experiments two workers (Dott & Glover) made counts independently on all the smears.

Three systems of counting were used:

(*a*) The number of dead spermatozoa in each microscopic field was written down as soon as the field had been counted, but these numbers were not summed until all the smears of the experiment had been examined.

(*b*) Only the total number of dead spermatozoa in each count of 100 cells was written down; the two counts on each smear were made in quick succession.

(*c*) The same as (*b*) except that each smear was counted once and these results were put out of sight before the second set of counts was begun.

Systems (*a*) and (*b*) were in use by the two workers at the start of these investigations; system (*c*) was adopted at a later stage when there was found to be a difference between the results given by systems (*a*) and (*b*).

Biometrical methods

The proportions of dead cells were transformed by the angular transformation; this transformation is particularly useful with binomial proportions because the variance of the angles is independent of the mean angle and has, moreover, a theoretical value which can be used to test whether the original proportions do follow a binomial distribution. For counts of 100 spermatozoa the theoretical variance is 8·207. In these analyses the observed variance has regularly exceeded this value, but the transformation has been retained as probably giving a rather better variable for analysis than are the untransformed proportion dead.

As two workers made counts in most of the experiments, the main analysis used has been a two-way classification analysis of variance, the classifications being by counter and smear. The smear classification is divided hierarchically into smears within subsamples, subsamples within replicates, and replicates, each term in the hierarchy being tested against that immediately below it (or any term from which this does not differ significantly). The variation between counts within counters and smears, which is outside the main two-way classification, can be used to test the counter × smear interactions, thus providing a valuable check on counting technique.

Experiments with ram semen

1. The object of this experiment was to study the effect on the proportion of dead cells of the time the spermatozoa were exposed to the stain before the smears were made. The exposure times used were 0, 1, 2, 3, 4, 5, 15, 30 and 60 minutes. Two subsamples were prepared, one by each experimental worker, and two smears were made at each time from each subsample. Each worker made three counts on each smear, one worker using counting system (*a*) and one using system (*b*). As will be shown below, counts using system (*b*) have been discarded because of the remarkably small differences within the triplicates.

Table 1. *The effect on the proportion dead of the time the spermatozoa were exposed to the stain before the smears were prepared*

Interval (min.)	% dead	Counts mean square	Smears mean square
0	25·6	8·58	199·70
1	26·6	6·45	54·55
2	24·2	27·75	72·45
3	32·7	12·82	23·23
4	23·4	20·43	28·87
5	39·4	26·13	31·84
15	39·3	14·54	15·65
30	43·2	9·63	3·83
60	51·3	8·49	22·00

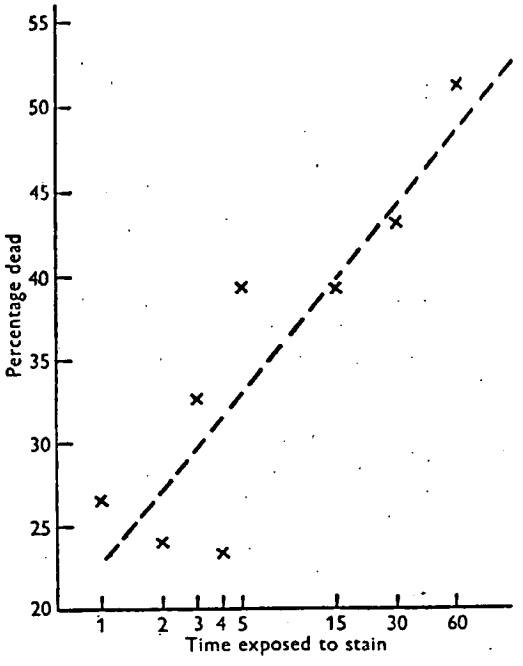

Fig. 1. The effect on the proportion of dead spermatozoa of the time the semen was exposed to the stain before the smears were made.

The results of this experiment (based on system (*a*) counts only) are summarized in Table 1, and in Fig. 1 the percentage of dead spermatozoa is plotted against the logarithm of the time in stain. This plot is approximately linear, and shows that when the

me in the stain is doubled the proportion of dead permatozoa increases by about 4 %; this regression significant at the 1 % level. The variation with me of the between-counts mean squares appears be random, but the between-smears mean squares ecrease significantly as the time in the stain creases ($P < 0{\cdot}05$). This suggests that the semen-ain mixture does not become homogeneous until veral minutes after it has been prepared.

The pooled estimated of the between-counts ariation was 14·99 on 72 degrees of freedom; com-aring this with the theoretical variance for trans-rmed binomial proportions gave a χ^2 of 131, signi-cant at the 0·1 % level. The corresponding mean quare and χ^2 for system (*b*) counts were 5·73 and), also on 72 degrees of freedom. The difference etween these two mean squares, tested by a two-ded *F*-test, was significant at the 1% level. At e time this experiment was carried out, these sults were taken to mean that system (*a*) counts ere less reliable because the variation was larger an expectation but, as the results of later experi-ents will show, it was in fact system (*b*) counts at were questionable.

2(*a*). This experiment was designed to study the fect of diluting ram semen on the proportion of ead cells and on the variability of the counts; lution of bull semen has been found to have no fect on these characteristics (Campbell *et al.* 1953). ve subsamples were prepared with whole semen d five with semen previously diluted 1 : 5 with trate buffer solution. Two smears were prepared om each subsample and four counts were made each smear by one worker using counting system); four counts rather than two were made because appeared in Exp. 1 that the counts variation btained by this system was larger than expected, that a more precise estimate of this variation was unted.

The mean proportion dead in the subsamples ade with whole semen was 39·3 %, and the pro-rtion for subsamples made with diluted semen as 33·4 %. This difference, although large, was not gnificant. The counts mean squares for the two ts of smears were 27·40 (whole semen) and 15·75 iluted semen), each on 30 degrees of freedom; the fference between these, tested by a two-sided -test, was not significant. The pooled mean square, ·57, gave a highly significant χ^2 of 158 on 60 egrees of freedom when compared with the theo-tical variance.

The between subsamples variation was not fferent from the between smears variation, but is was significantly larger than the between unts variation.

2(*b*). In making the counts in the previous experi-ents, 'clumps' of spermatozoa, most of which ere stained, were observed scattered over the smears. It was thought that the presence of such clumps in the fields counted might have led to the increased counts variation found with system (*a*), and to examine this problem the smears prepared for experiment 2(*a*) were recounted using the same system but omitting the spermatozoa forming clumps.

The mean proportions dead in the clumpless counts were 29·4 % for the smears made using whole semen and 22·6 % for those made using diluted semen. These were respectively 9·9 % and 10·8 % less than the values obtained in Exp. 2(*a*) by overall counts on the smears. The between counts mean square (14·65 on 20 degrees of freedom) was rather smaller than the value for the overall counts, but this difference was not significant.

3. In this experiment overall and clumpless counts were made on smears prepared from diluted and whole semen; four subsamples were made with diluted semen and four with whole semen, and two smears were made from each subsample. Each worker made duplicate counts, both overall and clumpless, on all the smears; the overall and clumpless counts were made as far as possible on the same fields.

Table 2. *The mean proportions of dead spermatozoa and the between counts mean squares, for counts including or ignoring the clumps of cells, on smears from whole or diluted semen*

	Overall		Clumpless	
	Dott	Glover	Dott	Glover
	Whole semen			
Mean (%)	33·7	28·7	27·0	20·1
Counts mean square	14·6	12·7	15·7	7·6
	Diluted semen			
Mean (%)	38·0	30·9	29·4	24·6
Counts mean square	16·1	53·5	23·4	23·8

In addition, an attempt was made to find the cause of the differences in the earlier experiments between the counts variations given by systems (*a*) and (*b*). It was suggested that when system (*b*) was used the result of the second count might unconsciously be made near to that of the first count, thus producing artificially small variations. This form of bias could not arise with system (*a*) because the total number dead in the first count was not known until after the second count had been completed. To test this suggestion system (*c*) was used in place of system (*b*) in this experiment.

The results of this experiment are summarized in Table 2. The mean proportions dead in the overall and clumpless counts were 32·8 % and 25·3 %; the difference between these values, which is highly significant, agrees reasonably well with the value obtained in experiment 2(*b*). The mean proportions in smears made with whole and diluted semen were

·4 % and 30·7 %; this difference is not significant nd is indeed of opposite sign to that found in xps. 2(*a*) and 2(*b*). The difference in the mean roportion dead as estimated by the two counters as 5·9 %, Dott's counts giving the higher mean; his difference is highly significant. None of the ounters × smears interactions was significant, hich shows that this counter difference was regular roughout the whole experiment.

The between-counts mean squares were homoeneous throughout the experiment; in particular, he values for the two counting systems were not gnificantly different, system (*c*) giving slightly the rger variation. As in Exp. 2, the mean square r the overall counts was larger than that for the umpless counts (24·22 as compared with 17·60, oth on 32 degrees of freedom) but again this ifference was not significant. A pooled value over verall and clumpless counts cannot, however, be sed because the two types of count were made over pproximately the same fields and so are not ndependent.

The between subsamples variation was signicantly larger than the between-smears variation, ut this did not exceed the between-counts variation.

These results show that the variation between ounts is much larger in ram semen than the value btained with bull semen by Campbell *et al.* (1953). n attempting to explain both this large variation n ram semen counts and the difference of mean etween counters mentioned above, the results of n earlier investigation are of interest, and this will ow be described.

4. In this experiment the numbers of partially nd completely stained spermatozoa were recorded eparately, spermatozoa being classed as partially tained if either

(*a*) they had a greyish appearance instead of he normal white or pink, or

(*b*) the posterior portion of the head was disinctly stained red whilst the anterior portion was nstained; in this case the acrosome might retain typical 'live' appearance (Hancock, 1952).

Whole semen was used in preparing the subsamples; ight smears were made from a subsample conaining stain dissolved in distilled water, and eight rom a subsample containing stain dissolved in itrate buffer solution. Three counts were made by ystem (*a*) on each smear.

The mean proportions dead are given in Table 3. In the distilled water stain, 36·6 % of the spermatozoa appeared stained but only 11 % of these were completely stained. Using stain dissolved in citrate buffer solution, 40·4 % of the spermatozoa were stained and 45 % of these were completely stained. This difference between stain solvents in the proportion of spermatozoa that were completely stained was significant, but that between the total proportions dead was not significant.

The variation between counts was the same with both stain solvents and in the counts of partially and completely stained spermatozoa. The mean square for the counts including all stained spermatozoa (partial and complete) was 29·61 on 32 degrees of freedom. The subsamples mean square with the stain dissolved in distilled water was larger than that with the stain dissolved in citrate buffer solution ($F = 3·24$ on 6,6 degrees of freedom) but this difference was not significant.

Experiments with boar semen. Two experiments were carried out, both to the same design but using different samples of semen, to determine the reproducibility of live-dead counts. In both experiments each worker prepared three subsamples, using stain dissolved in distilled water, and made two smears from each subsample. Both workers made duplicate counts on all the smears, all the spermatozoa in the fields examined, whether forming clumps or not, being included in the counts; one worker used system (*a*) and one system (*c*).

Table 3. *The mean percentages of partially and completely stained spermatozoa in counts on smears prepared using two different stain solvents*

	Degree of staining	
Stain solvent	Partial	Complete
Distilled water	32·3	4·2
Citrate buffer solution	22·3	18·1

The results of the two experiments agreed well in the estimates they gave for the reproducibility of the different stages of the smearing and counting technique. In the overall analysis the between counts mean square, 13·30, was significantly greater than the expected value; the variation between subsamples was not larger than that between smears, but this was significantly greater than the counts variation. The mean proportions dead obtained by the two counters were:

	Dott (%)	Glover (%)
Exp. 1	20·1	16·9
Exp. 2	19·2	18·6

The overall difference between counters, 1·9 %, was not significant. None of the counters × smears interactions was significant.

Experiments with bull semen. In the experiments described above the counts variation was much larger than that reported by Campbell *et al.* (1953); these workers, moreover, did not make any reference to clumping. For these reasons we thought it worth while to carry out a small experiment to test their findings.

In this experiment two samples of semen from different bulls were used, and four subsamples were prepared from each, using stain dissolved in distilled water. Two smears were made from each subsample, and both workers made duplicate counts on all the smears, using the same counting systems as in the experiments with boar semen.

The results of the two experiments agreed well in the estimates they gave for the reproducibility of the various stages of the smearing and counting technique. In the overall analysis the between counts mean square, 11·13, although larger than that obtained by Campbell *et al.* (1953), 8·51, did not significantly exceed its theoretical value. The variation between subsamples was not larger than that between smears, but this was significantly greater than the between counts variation. The mean proportions dead in two experiments were:

	Dott (%)	Glover (%)
Exp. 1	24·0	20·9
Exp. 2	22·0	17·8

The overall difference between counters, 3·6 %, was highly significant; none of the counters × smears interactions was significant.

Clumps of spermatozoa were very uncommon on the smears in both the boar and bull experiments.

DISCUSSION

Random variation is introduced into live-dead counts of spermatozoa at three main stages of the sampling process: preparing the subsamples (semen-stain mixtures), making the smears and counting the stained and unstained cells. These three stages have been separated in the analysis of variance hierarchy, and will be considered separately in the first part of this section.

Variation between counts on the same smear

The main finding of these investigations is that, in smears made from ram or boar semen, the variation between counts is significantly larger than the value expected for binomial proportions. This shows that the underlying assumption of the binomial distribution—that the stained and unstained cells are distributed uniformly and independently over the smear—is not satisfied in smears made with semen from these two species. Although the present results with bull semen do not differ significantly from those of Campbell *et al.* (1953), we consider that, in view of the results with ram and boar semen, it is probably better to use the estimated counts mean square in place of the theoretical value adopted by those authors.

One factor which may be expected to have a large effect on the uniformity of the distribution of the cells is the occurrence of 'clumps' of stained spermatozoa. This phenomenon, which is much more common in smears of ram semen than in those prepared with boar or bull semen, has been shown to have a pronounced effect on the estimate of the proportion of dead spermatozoa, there being a much higher proportion of stained spermatozoa in the clumps than in the rest of the smears. The identification of clumps was somewhat arbitrary, and this may explain the fact that omitting the clumps (ram, Exp. 3) did not significantly reduce the counts mean square. Furthermore, attempts to break up the clumps by diluting the semen before making the subsamples had no significant effect on the estimated live-dead proportion or on the counts variation.

The existence of clumps of stained spermatozoa does not necessarily mean that dead spermatozoa tend to come together; an alternative explanation is that cells which happen to lie close to each other are more likely to appear stained because the smear will tend to be thicker at this point. The choice between these two explanations is important because they lead to different conclusions as to the best method of estimating the proportion dead. If dead cells tend to cohere, the clumps must be included in the count, otherwise the estimate obtained will be too low; if on the other hand cells that happen to be close together are more likely to appear stained, the clumps should be omitted from the counts, as their inclusion would make the estimate too high. The results of the present experiments can give no guidance about the choice between the two explanations, but unpublished results show that the thickness of the smear has no effect on the estimate of the proportion stained. Moreover, Hancock & Shaw (1955) have found that the proportion of dead cells can be estimated by scoring the amount of agglutination in a suspension of semen and buffered formal saline, which shows that, at least in the conditions of their experiments, dead spermatozoa do form clumps. These findings suggest that the first explanation is correct, which means that counts to estimate the live-dead proportion must include all the spermatozoa in the field of view; the practice of selecting fields that are easy to count (i.e. that do not contain clumps of cells) may lead to entirely unreliable estimates.

Even when this precaution was applied in the later experiments of this series, there were significant differences between the estimates obtained by the two experimental workers. This may be due in part to difficulties in the interpretation of 'stained' and 'unstained' when many of the spermatozoa are only partially stained. The results of Exp. 4 with ram semen show that when the stain is dissolved in distilled water, for every spermatozoon which takes the stain completely another eight take it to a lesser but still visible degree. It was observed, although

no counts were made, that boar and bull spermatozoa showed better differentiation, with fewer partially stained cells than in the ram semen. This observation, together with the values of the counter differences in the mean proportion dead for the three species (5·9 % for ram, 1·9 % for boar and 3·6 % for bull), supports the hypothesis that the partial staining was partly responsible for this counter difference. Moreover, the counts mean square was larger in the ram (24·22) than in the other species (boar, 13·30 and bull 11·13), which suggests that the occurrence of partial staining also influences the precision of the counts. There are presumably species differences in the structure of the head membrane of the spermatozoon and in the way in which it degenerates when the cell dies.

A further difference was that between the two staining solutions. In the distilled water stain some of the spermatozoa took on a greyish appearance, but in the citrate buffer stain, whilst this effect occurred only rarely, some cells showed distinct staining of the posterior portion of the head whilst the anterior portion remained unstained. This latter effect has also been reported by Swanson & Bearden (1951). These differences between the solvents may be related to the difference of tonicity—Rothschild & Barnes (1954) have shown that 2·875 % citrate buffer solution containing sulphanilamide is isotonic with bull seminal plasma, so that the stain dissolved in citrate buffer solution will be hypertonic.

Variations between smears and between subsamples

Less information is available on these forms of variation than on that between counts, but in most experiments at least one was significantly large. The results for the different species are not the same, the subsamples variation being significant for the ram but not for the boar or bull, whilst the slides variation was significant for these species but not for the ram. Rather, however, than postulate different forms of heterogeneity for the different species, it seems better to regard both types of variation as important in every case; the tables in the next section have been computed on this basis.

The existence of these variations means that the distribution of live and dead spermatozoa was not uniform in the ejaculates. This is not surprising, in view of the presence of clumps of dead cells on the smears and suggests that this clumping may be very marked in ejaculated semen or even in the epididymis. In this connexion, Blom (1950) has suggested that 'barriers' of dead cells may actually be the cause of the typical wave motion of whole semen. Walton (1952) does not agree with this interpretation of wave motion and Taylor (1951) has shown that spermatozoa will tend to swim with their tails in phase because this reduces the dissipation of energy into the surrounding fluid. Taylor does not appear, however, to have considered the effect of the presence of dead cells on the movement of the living.

The difference between the subsamples mean squares obtained when distilled water and citrate buffer solution were used as stain solvents, although not significant, suggests that it may be possible to reduce the subsamples and smears variation by choice of a suitable solvent.

Practical instructions

The purpose of this section is to summarize the findings of this series of experiments in so far as these indicate methods of controlling the accuracy of live-dead counts. The interval between preparing the subsample and making the smears should be kept constant; five minutes appears to be a satisfactory time. The importance of keeping this factor constant with bull semen has been noted by Brochart (1953).

Duplicate counts, either on the same or different smears, need to be kept free from any unconscious bias; this can be ensured by using either counting system (*a*) or system (*c*).

It is necessary to decide beforehand what 'stained' and 'unstained' mean and to use a stain solvent that reduces as far as possible the proportion of partially stained spermatozoa. Citrate buffer solution is in this respect the better of the two tested in these experiments, but this solvent has the disadvantage that unless the staining solution is fresh the smears are liable to crack when mounted.

The numbers of spermatozoa that should be counted to achieve a given accuracy are given for the three species in Tables 4, 5 and 6. The accuracy is measured by the standard error of the mean proportion dead; this proportion is probably approximately normal for the values that are usually encountered, but may well be non-normal for values below 10 % or above 90 %, so that the standard error should only be used as a rough indication of the precision in these cases. More exact measures of precision might be devised after studying the distribution of the estimated proportion dead, but this would require a very lengthy investigation and would need to be linked with more precise measurements of the subsample and smear variations.

The standard errors given in the tables may be used to determine approximate fiducial limits for the proportion dead, as in the following example. Suppose that in a count of 400 ram spermatozoa, each 100 being counted on a separate smear made from a separate subsample, the observed proportion dead is 18·2 %. The standard error corresponding to such a count is shown in Table 4 to lie between 3·6 % and 4·1 %, say 3·9 %. The 95 % fiducial limits for the true proportion dead are $(18{\cdot}2 \pm 2 \times 3{\cdot}9)$ %.

Table 4. *The standard errors of estimates of the proportion dead (%) in ram semen for various numbers of subsamples, smears per subsample, and counts (each of 100 spermatozoa) per smear*

(If the percentage dead is more than 50, use the percentage of live spermatozoa for calculations and the same table. For any given number (n) of counts, maximum accuracy is obtained by making n subsamples, one smear per subsample and one count per smear.)

No. of subsamples	No. of smears	No. of counts	True proportion dead (%) 5	10	15	20	25	30	40	50
1	1	1	4·4	6·1	7·3	8·2	8·8	9·3	10·0	10·2
1	1	3	3·2	4·4	5·3	5·9	6·4	6·8	7·2	7·4
1	1	6	2·8	3·9	4·6	5·2	5·6	5·9	6·4	6·5
1	2	1	3·6	4·9	5·8	6·5	7·1	7·5	8·0	8·2
1	2	3	2·8	3·9	4·6	5·2	5·6	5·9	6·4	6·5
1	3	1	3·2	4·4	5·3	5·9	6·4	6·8	7·2	7·4
1	6	1	2·8	3·9	4·6	5·2	5·6	5·9	6·4	6·5
2	1	1	3·1	4·3	5·1	5·8	6·2	6·6	7·1	7·2
2	1	3	2·3	3·1	3·7	4·2	4·5	4·8	5·1	5·2
2	3	1	2·3	3·1	3·7	4·2	4·5	4·8	5·1	5·2
3	1	1	2·6	3·5	4·2	4·7	5·1	5·4	5·8	5·9
3	2	1	2·1	2·8	3·4	3·8	4·1	4·3	4·6	4·7
4	1	1	2·2	3·0	3·6	4·1	4·4	4·7	5·0	5·1
6	1	1	1·8	2·5	3·0	3·3	3·6	3·8	4·1	4·2

Table 5. *The standard errors of estimates of the proportion dead (%) in boar semen for various numbers of subsamples, smears per subsample, and counts (each of 100 spermatozoa) per smear.*

(If the percentage dead is more than 50, use the percentage of live spermatozoa for calculations and the same table. For any given number (n) of counts, maximum accuracy is obtained by making n subsamples, one smear per subsample and one count per smear.)

No. of subsamples	No. of smears	No. of counts	True proportion dead (%) 5	10	15	20	25	30	40	50
1	1	1	4·0	5·5	6·5	7·3	7·9	8·4	8·9	9·1
1	1	3	3·3	4·5	5·4	6·0	6·5	6·9	7·3	7·5
1	1	6	3·1	4·2	5·0	5·6	6·1	6·4	6·9	7·0
1	2	1	3·3	4·5	5·4	6·1	6·6	6·9	7·4	7·6
1	2	3	2·9	4·0	4·7	5·3	5·7	6·1	6·5	6·6
1	3	1	3·0	4·2	5·0	5·6	6·0	6·4	6·8	7·0
1	6	1	2·8	3·8	4·5	5·1	5·5	5·8	6·2	6·3
2	1	1	2·8	3·9	4·6	5·2	5·6	5·9	6·3	6·4
2	1	3	2·3	3·2	3·8	4·2	4·6	4·9	5·2	5·3
2	3	1	2·1	3·0	3·5	3·9	4·3	4·5	4·8	4·9
3	1	1	2·3	3·2	3·8	4·2	4·6	4·8	5·2	5·3
3	2	1	1·9	2·6	3·1	3·5	3·8	4·0	4·3	4·4
4	1	1	2·0	2·7	3·3	3·6	3·9	4·2	4·5	4·6
6	1	1	1·6	2·2	2·7	3·0	3·2	3·4	3·6	3·7

Table 6. *The standard errors of estimates of the proportion dead (%) in bull semen for various numbers of subsamples, smears per subsample, and counts (each of 100 spermatozoa) per smear.*

(If the percentage dead is more than 50, use the percentage of live spermatozoa for calculations and the same table. For any given number (n) of counts, maximum accuracy is obtained by making n subsamples, one smear per subsample and one count per smear.)

No. of subsamples	No. of smears	No. of counts	True proportion dead (%) 5	10	15	20	25	30	40	50
1	1	1	3·2	4·5	5·3	6·0	6·5	6·8	7·3	7·5
1	1	3	2·5	3·4	4·1	4·6	5·0	5·3	5·6	5·7
1	1	6	2·3	3·1	3·7	4·2	4·5	4·8	5·1	5·2
1	2	1	2·3	3·2	3·8	4·2	4·6	4·8	5·2	5·3
1	2	3	1·8	2·4	2·9	3·3	3·5	3·7	4·0	4·1
1	3	1	1·9	2·6	3·1	3·4	3·7	3·9	4·2	4·3
1	6	1	1·3	1·8	2·2	2·4	2·6	2·8	3·0	3·0
2	1	1	2·3	3·2	3·8	4·2	4·6	4·8	5·2	5·3
2	1	3	1·8	2·4	2·9	3·3	3·5	3·7	4·0	4·1
2	3	1	1·3	1·8	2·2	2·4	2·6	2·8	3·0	3·0
3	1	1	1·9	2·6	3·1	3·4	3·7	3·9	4·2	4·3
3	2	1	1·3	1·8	2·2	2·4	2·6	2·8	3·0	3·0
4	1	1	1·6	2·2	2·7	3·0	3·2	3·4	3·6	3·7
6	1	1	1·3	1·8	2·2	2·4	2·6	2·8	3·0	3·0

i.e. 10·4 % and 26·0 %; these values are such that if the true values lay outside them there would be a chance of less than 5 % of the value 18·2 % being observed. The limits are only approximate because the standard errors are computed on the assumption that the proportion dead used is the true value, rather than an observed value.

For any given total number (n) of counts, the maximum accuracy is obtained by preparing n subsamples and making one smear from each subsample and one count on each smear.

Counts should include all the spermatozoa in the field of view, and fields should be chosen at random without any attempt to select those that are easy to count.

Future work

The investigation of the effect of variations in the interval between preparing the subsamples and making the smear could usefully be repeated on further semen samples, both from the ram and from other species (the present study dealt only with one ram sample). Another form of time effect which preliminary observations suggest may be of some importance is that of the interval between smearing and counting.

Since the present experiments were undertaken, Bangham & Hancock (1955) have devised a new method for estimating the proportion of live and dead spermatozoa, which may in the future reduce the practical applications of differential staining. The physiological problems associated with live-dead counts will, however, still require study; among the most interesting of these are the nature and causes of partial staining and clumping. The results of Exp. 4 suggest that the former occurrence may be influenced by the choice of stain solvent, so that experiments in which the pH and tonicity of the stain solution were varied might help to elucidate this problem. Clumping, or the agglutination of dead spermatozoa, is already being investigated from another point of view by Dr J. L. Hancock.

SUMMARY

1. The proportion of stained spermatozoa in a ram semen-nigrosin-eosin mixture has been found to increase with the time of exposure to the stain. Five minutes is considered to be a suitable interval for practical use.

2. The value obtained for the proportion of dead cells can be considerably affected by the selection of fields that are easy to count; fields should be chosen at random.

3. The variation between counts on the same smear is much larger than that expected for binomial proportions. This may be due partly to the 'clumping' of the dead spermatozoa and partly to the difficulties of interpretation caused by the presence of many partially stained cells.

4. A significant difference was found between the estimates of the proportion dead obtained by the two experimental workers; this is thought to be due to a difference of interpretation of 'stained' and 'unstained'.

5. Tables are given by which to determine the approximate accuracy of counts made on a range of numbers of spermatozoa, smears and subsamples, separate tables being necessary for ram, boar and bull semen.

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STUDIES ON THE MALE REPRODUCTIVE SYSTEM

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THE HYPOTHALAMUS AND SEMINAL EMISSION

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SUMMARY

1. Stimulation of the hypogastric nerves in adult male rabbits under sodium pentobarbitone anaesthesia produced immediate contractions of the cauda epididymidis, vas deferens and seminal vesicle, with emission of semen. Kymographic records were obtained of the contractions of the seminal vesicle.

2. Intravenous injections of 1–5 μg adrenaline gave similar effects, but with latencies of 7–30 sec. Measured by its action on the seminal vesicle, adrenaline was two to five times more potent than noradrenaline.

3. Electrical stimulation of the dorsal, lateral or posterior areas of the hypothalamus evoked an immediate contraction of the seminal vesicle followed by a delayed contractile effect. Contractions of the cauda epididymidis and vas deferens also occurred.

4. The immediate response of the seminal vesicle to stimulation of the hypothalamus was abolished by section of the hypogastric nerves, and the delayed effect by adrenalectomy. After these interferences the two types of response could be simulated by stimulation of the peripheral end of the cut hypogastric nerves, and by injection of 1–5 μg adrenaline, respectively.

5. Neither electrical stimulation of the neurohypophysis, nor injection of oxytocin or vasopressin induced a contraction of the seminal vesicle.

6. The results are discussed with reference to the influence of sexual excitation at coitus on the volume and quality of ejaculated semen.

Emission of spermatozoa and seminal plasma into the urethra involves the contraction of smooth muscle in the male accessory sex organs, and is therefore under the motor control of the autonomic nervous system. There are firm grounds for the view that the lumbar sympathetic outflows are those chiefly concerned [Langley & Anderson, 1895]. Ejaculation, on the other hand, which involves the forcible ejection of semen from the penis, depends on the action of striated musculature innervated by somatic motor fibres in the pudendal nerves [Semans & Langworthy, 1938]. Both processes normally occur in association as reflex effects of stimuli from the glans penis during coitus. It seems likely, however, that emission and ejaculation may be affected by higher levels of the central nervous system, e.g. those whose activity reflects the emotional excitation of the animal. Recent work in the female rabbit has shown that stimulation of the sympathetic area of the hypothalamus induces characteristic effects on the contractile mechanisms of the mammary gland and uterus [Cross, 1955, 1957]. The present paper reports on the influence of similar excitation of the hypothalamus on seminal emission in rabbits.

In this study observations have been made on various accessory sex organs, but the main attention has been focused on the median bilobed sac, which we shall refer to as the 'seminal vesicle'. This structure has been variously called the uterus masculinus, prostate and prostatic vesicle (see Gruber [1933]). The organ actually comprises the glandula vesicularis, the paired glandulae seminales and the prostate proper.

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Davies & Mann [1947*a*] showed that the glandula vesicularis and glandula seminalis were derived from a common diverticulum of the Wolffian duct. On these grounds and from biochemical findings [Davies & Mann, 1947*b*], they were regarded as the homologue of the paired seminal vesicles in other animals.

MATERIALS AND METHODS

Twenty adult male rabbits weighing from 2·4 to 4·0 kg and of mixed strain were used in the experiments. All the animals were sexually experienced. They were housed in wire-mesh cages, maintained at 68° F and given constant access to drinking water and compound pellets (diet 18, Oxo Ltd.).

The animals were anaesthetized by intravenous sodium pentobarbitone (Veterinary Nembutal solution, Abbott Laboratories) as previously described [Cross, 1955]. The urinary bladder was emptied by manual expression to facilitate subsequent exposure of the seminal vesicle. With the animal in the supine position, a laparotomy incision was made from the umbilicus to the brim of the pubis. The urinary bladder was retracted posteriorly to reveal the ampullae of the vasa deferentia between which the seminal vesicle is situated. The blind end of the vesicle, now facing upwards, was attached by a length of nylon thread to a simple lever recording on a kymograph. The intestines were packed away from the site by pads of cotton-wool wrung out from normal saline at 37° C. To reduce artifacts resulting from displacement of the vesicle by the passage of faeces, the adjacent portion of the colon and rectum was emptied and a ligature tied around the colon proximally. The exposed organs were moistened periodically with warm saline.

To locate the hypogastric nerves it was only necessary to elevate the terminal portion of the colon and scrutinize the region of the posterior mesenteric artery for the ganglion giving origin to the nerves, i.e. the posterior mesenteric ganglion. Stimulation of the hypogastric nerve was effected by a bipolar electrode, using as the stimulus 50 cyc/sec sine wave alternating current at 1–3 V (root mean square value). The number and disposition of the hypogastric nerves varied slightly in different rabbits. The stimulating electrode was useful in helping to locate the nerves and in testing the effectiveness of section, by the absence of a response in the vesicle or vasa deferentia on stimulation proximal to the point of nerve section.

For stimulation of the hypothalamus the fore part of the body was pronated and the head orientated in the stereotaxic instrument. The technique of stimulation with a glass-insulated platinum wire unipolar electrode was essentially the same as that described by Cross & Harris [1952]. The bare tip of the electrode was 0·5 mm long, and from this earlier work it is probable that the spread of stimulus is not more than 0·5 mm at the voltage employed, i.e. 1·0 V.

To perform adrenalectomy during an experiment the laparotomy incision was extended to the xiphisternum and the intestines collected outside the wound between pads of cotton-wool, soaked in warm saline. The *right* adrenal was first freed as far as possible from adherent connective tissue and the vena cava. It was then firmly grasped with curved forceps as close to the vena cava as possible and seared off with a thermocautery. The *left* adrenal was removed, somewhat more readily, by a similar procedure. The intestines were returned to the abdomen and the extended incision

closed with Michel clips. This operation took about 25 min. In some of the animals the right adrenal gland was extirpated at a preliminary operation. In these cases the gland was exposed through an incision posterior to the last rib by reflecting the right kidney medially. The adrenal gland was then removed as before. The muscle and skin layers were closed separately by nylon mattress sutures. At the time of the experiment, a few minutes only were needed for the removal of the remaining left adrenal gland, and this could be done without withdrawing the animal's head from the stereotaxic instrument. In all cases the adrenal sites were examined *post mortem* to check the completeness of adrenalectomy.

All drugs were given in normal saline into an ear vein. The products used were adrenaline tartrate (containing 1:1000 adrenaline; Burroughs Wellcome Ltd.); 'Levophed' (containing 1 mg/ml. noradrenaline as the bitartrate; Bayer Products Ltd.); 'Pitocin' and 'Pitressin' (purified oxytocic and vasopressor principles of the posterior lobe of the ox pituitary, containing 10 i.u./ml.; Parke Davis and Co.).

At the conclusion of each experiment the animals were killed by a rapid intravenous injection of Nembutal. For histological identification of electrode sites, the brains were perfused *in situ* with 20 ml. aqueous Bouin's fluid through each carotid artery. They were later removed from the skull and placed in 5 % formol-saline. Blocks of tissue containing the hypothalmus were then prepared, washed in running tap water for 24 hr, then placed in 25 % ethanol. Frozen sections were cut horizontally at 100 μ, i.e. at right angles to the plane of the electrode tracks. The sections were mounted serially and stained with thionin. Since care was taken during the experiments not to depress the electrode deeper than the points of stimulation, the sites of the electrode tips were identified without difficulty (see Plate).

RESULTS

Contraction of the seminal vesicle as an index of seminal emission

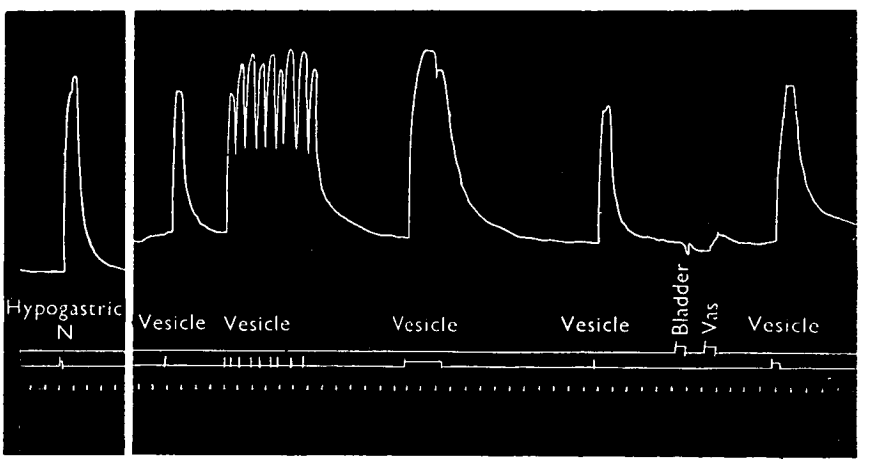
Emission of semen into the pelvic urethra involves the contraction of the cauda epididymidis, vas deferens, seminal vesicle and other male accessory glands. Since the classical work of Langley & Anderson [1895] on the functional innervation of the male generative organs in the rabbit, it has come to be generally accepted that the lumbar sympathetic outflows comprise the main motor pathway in seminal emission. As a first step in this investigation, therefore, it was decided to observe the effects of stimulation of the hypogastric nerves on the male organs at laparotomy.

(*a*) *Effect of stimulation of the hypogastric nerves*

The most conspicuous effect of stimulating the hypogastric nerves or the posterior mesenteric ganglion in eight rabbits was an immediate and intense contraction of both vasa deferentia. In one case only it was found possible to activate the two vasa independently by stimulation of the left and right hypogastric nerves. When the urinary bladder was retracted posteriorly to bring the seminal vesicle into view, it was seen that a powerful contraction of the latter accompanied the responses of the vasa to stimulation of the hypogastric nerve. The vesicle contracted to about a quarter of its original size and then slowly relaxed. The total duration of the response to a stimulus lasting 1–2 sec was commonly about 1 min. When the vesicle was full of

secretion the latter was forcibly expressed by the contraction. In one experiment drops of seminal fluid were emitted from the end of the penile urethra following hypogastric nerve stimulation.

To observe the epididymis, the testis was exposed either by incising the scrotum or by pushing it through the inguinal canal so as to lie close to the seminal vesicle. Stimulation of the hypogastric nerves induced a contraction of the cauda epididymidis that involved the epididymal tubules of larger calibre. The head and body of the epididymis were not visibly affected. When in one animal the vas deferens was cannulated with polythene tubing near to the epididymis, stimulation of the hypogastric nerves resulted in the ejection of epididymal fluid into the cannula.



Text-fig. 1. Contraction of the seminal vesicle after direct stimulation, and stimulation of the hypogastric nerve. Note that stimulation of the urinary bladder and vas deferens produced only small deflexions of the tracing. Time intervals 30 sec.

Of these various motor components of seminal emission, the seminal vesicle seemed to offer the best prospect for more detailed study, since kymographic records of its contractions were readily obtainable by the use of a simple lever system. Text-fig. 1 shows part of the record of an experiment in which the utility of the method was demonstrated. A 5 sec stimulus to one hypograstric nerve elicited a rapid contraction of the seminal vesicle, which did not fully relax for more than 1 min. Direct stimulation of the vesicle produced a similar response, the size of which depended on the duration of the stimulus. A partial tetanus of the organ could be produced by repeated stimuli. Similar direct excitation of the urinary bladder and vasa deferentia, though inducing contractions of these structures, caused only minor fluctuations in the tracing from the seminal vesicle. Nevertheless, in many experiments an irregular base line was recorded. This was due to two principal factors. First, the vesicle frequently exhibited spontaneous contractile activity; second, it was not always possible to prevent slight displacement of the organ by peristaltic movements of the colon. However, the base-line fluctuations seldom caused difficulty in interpreting the kymographic records as experimental stimulation induced much more pronounced effects (Text-figs. 2, 3).

(*b*) *Effect of adrenaline and noradrenaline*

When experiments were begun on the effects of adrenaline on the male organs it was soon found that the seminal vesicle was highly responsive to small intravenous doses of the hormone. Observations in ten rabbits showed that injection of 1–5 μg adrenaline resulted in contractions of the vesicle resembling those provoked by hypogastric nerve stimulation. The latency was longer, viz. 7–30 sec, and usually a pronounced vasoconstriction occurred in the organ. The responses were readily reproducible and their magnitude varied with the dose. The latency tended to be longer with the smaller doses. In one experiment, for example, injection of 1 μg adrenaline gave a small response after 18 sec, 5 μg gave a large response after only 7 sec and 3 μg gave a response of intermediate size after 8 sec. A similar effect with doses of 5 and 2 μg adrenaline is illustrated in Text-fig. 3. The latencies of these two responses were 10 and 25 sec respectively.

Injection of noradrenaline caused contractions of the seminal vesicle which were indistinguishable from those produced by adrenaline. When, however, the effects of the two amines were compared in six experiments, adrenaline always proved the more powerful excitant to the vesicle. Over the dose range of 1–10 μg, adrenaline appeared to be two to five times more active than noradrenaline.

In addition to the response of the seminal vesicle, contractions of the cauda epididymidis and vas deferens often resulted from the injection of adrenaline or noradrenaline. However, these effects were neither as marked nor as reproducible as the response of the vesicle. Moreover, the contractions produced in these organs by adrenaline were much less intense than those induced by stimulation of the hypogastric nerves. Since stimulation of the sympathetic area of the hypothalamus could be expected to evoke a secretion of adrenaline from the adrenal medulla as well as an excitation of fibres in the hypogastric nerves, the seminal vesicle seemed particularly suitable as a test organ for studying hypothalamic influences on seminal emission.

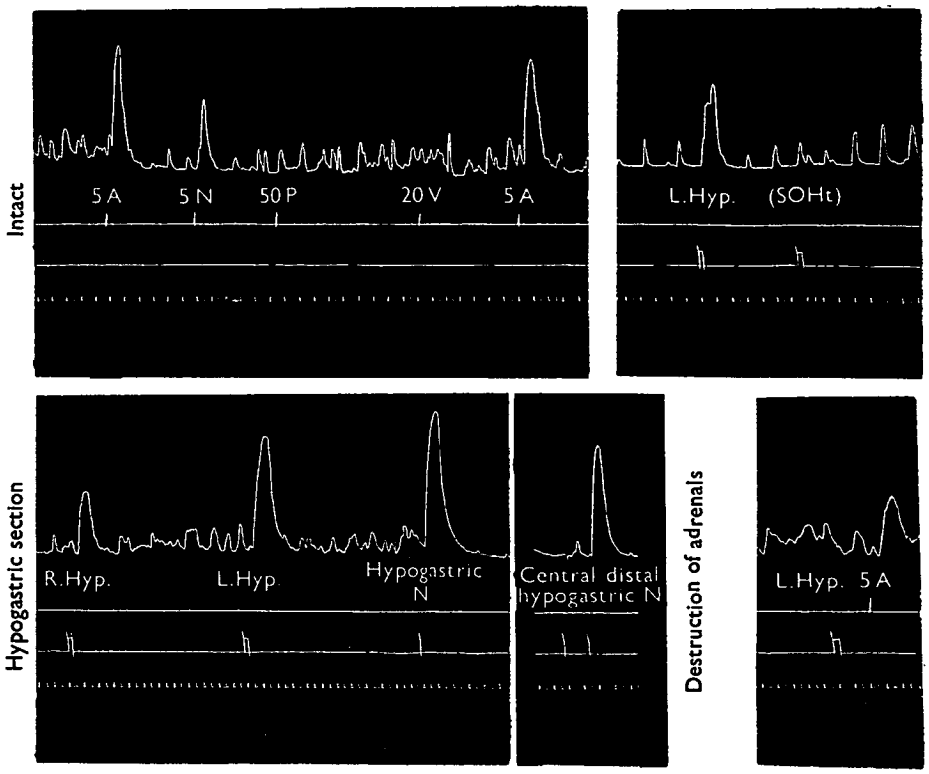
Response of the seminal vesicle to stimulation of the hypothalamus

Evidence that seminal emission may be induced by stimulation of the hypothalamus was obtained in fourteen rabbits. As a routine, the stimulating electrode was inserted 1·5 mm to the left or right of the bregma and racked down through the brain to a depth of 1·5 cm from the surface of the skull. This procedure was adopted in order to bring the uninsulated tip of the platinum wire electrode into the region of the hypothalamus, stimulation of which has been found in previous work to provoke a sympathetic discharge characterized by dilatation of the pupil, exophthalmos and the release of adrenaline from the adrenal medulla [Cross, 1955, 1957].

In one subject a testis was exposed and the vas deferens cannulated close to the cauda epididymidis with a short length of narrow-bore polythene tubing containing a small quantity of normal saline. Stimulation of the sympathetic zone of the hypothalamus for 15 sec induced a contraction of the cauda associated with a clearly visible emission of spermatozoa into the saline in the cannula. The effect was obtained several times.

In all other animals contractions of the seminal vesicle were recorded as the index of seminal emission. In every case appropriate stimulation of the hypothalamus

elicited a response of the vesicle comparable with the effects of stimulation of the hypogastric nerve and injections of adrenaline. Contractions of the vas deferens and cauda epididymidis were commonly seen to accompany the response of the vesicle and, in one instance, a vasoconstriction and subsidence of a previously erect penis was noted. The responses of the vesicle to hypothalamic stimulation were readily repeatable; as many as nine were evoked by successive stimulations in a period of $1\frac{1}{4}$ hr. Altogether sixty-two positive responses were recorded in the thirteen animals.



Text-fig. 2. Effect of section of hypogastric nerves and adrenalectomy on response of the seminal vesicle to hypothalamic stimulation. First part of the records shows spontaneous contractions of the vesicle. I.v. injection of 5 μg adrenaline (5A) caused a greater response of the vesicle than 5 μg noradrenaline (5N). 50 mu. Pitocin (50P) and 20 mu. Pitressin (20V) were without effect on the vesicle. Stimulation of the infundibular stem (SOHt) gave no response, but stimulation of the left perifornical area (L. Hyp) elicited a response after a latency of 5 sec. Following section of the hypogastric nerves the response of the vesicle to stimulation of the same hypothalamic site (L. Hyp.) had a latency of 24 sec, and stimulation of the dorsal hypothalamus just lateral to the right paraventricular nucleus (R. Hyp.) evoked a smaller response after a latency of 28 sec. Stimulation of the peripheral end of a cut hypogastric nerve, but not the central end, induced an immediate contraction of the vesicle. When both adrenals had been destroyed, hypothalamic stimulation had no effect, although the vesicle still responded to injection of 5 μg adrenaline. Time intervals 1 min.

With the exception of three animals whose hypogastric nerves had been sectioned before the experiment (see below) the contractile response of the seminal vesicle consisted of two phases. The initial contraction (referred to below as the 'quick response') began within a few seconds of the onset of stimulation. Then, usually before the vesicle had fully relaxed, one or more further contractions occurred. These

secondary contractions invariably commenced after the end of stimulation, and had a latency of up to 55 sec from the onset of stimulation (called, for convenience, 'slow response'). Typical effects of hypothalamic stimulation on the seminal vesicle are shown in the Plate.

Histological reconstruction of the electrode tracks showed the points at which stimulation produced a response to be located in the dorsal hypothalamus adjacent to the paraventricular nucleus, in the perifornical and lateral hypothalamic areas, or in the posterior hypothalamus in the vicinity of mammillothalamic tracts. Macrophotographs of sections through the hypothalamus showing the position of the electrode tips in five animals are reproduced in the Plate.

Since in previous work similar stimulation of the hypothalamus in the rabbit was shown to release neurohypophysial hormones [Cross, 1955, 1957], it was felt necessary to determine whether such a mechanism might contribute to the response of the seminal vesicle. Accordingly, in three rabbits the infundibular stem was stimulated. This procedure did not appear to influence the vesicle. Moreover, 20 mu. vasopressin likewise had no effect when injected in one animal; while in five rabbits injection of 50 or 100 mu. oxytocin failed to elicit a contractile response of the organ (Text-fig. 2).

It seemed likely, therefore, that the effects on the seminal vesicle of excitation of the hypothalamus were due to the resulting sympathetic discharge. Furthermore, the time relations of these effects suggested the participation of both nervous and humoral mechanisms. The quick response occurred too soon to be due to release of medullary adrenaline, and was probably a direct nervous effect. The slow response, on the other hand, coming after the end of stimulation, could well be the result of secretion of adrenomedullary hormone. This interpretation was tested by studying the effects of hypogastric nerve section and adrenalectomy on the response of the vesicle to hypothalamic stimulation.

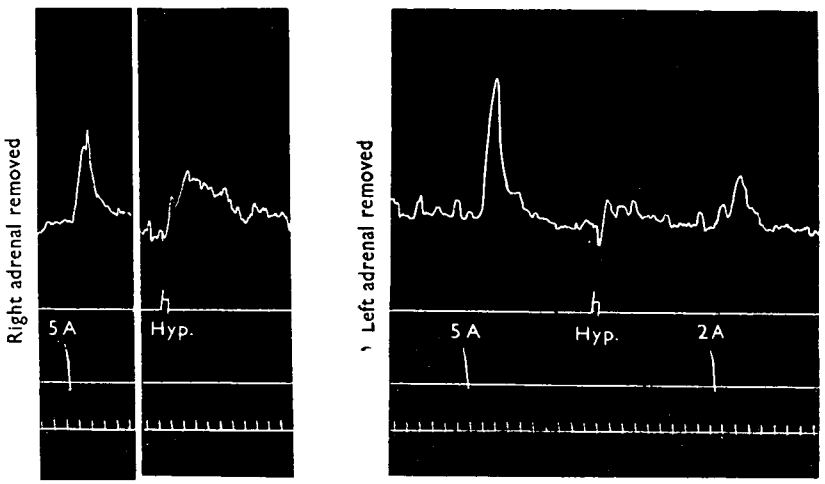
(a) Abolition of the quick response by section of the hypogastric nerve

In three animals, after recording a number of responses of the seminal vesicle to stimulation of the hypothalamus, the hypogastric nerves were sectioned and further stimulations performed. In each case the quick response was eliminated. In the intact animals the seminal vesicles responded after latencies of 2–7 sec; afterwards no response was seen in less than 24 sec from the onset of stimulation. However, the missing quick response could be imitated by stimulation of the peripheral end of the cut hypogastric nerve. Injection of adrenaline gave responses of the vesicle with latencies of up to 30 sec which thus more closely resembled the slow response still present after section of the hypogastric nerve.

Three other rabbits whose hypogastric nerves had been resected 2–3 weeks previously were also examined in this way. A contraction of the vesicle was produced by stimulation of the hypothalamus in each case, but the shortest latency observed was 15 sec. Adrenaline, when injected, evoked responses of the vesicle which had a somewhat shorter latency than those provoked by hypothalamic stimulation. These results, therefore, again indicated a nervous transmission of excitation from the hypothalamus to the seminal vesicle which was manifest in the quick response.

(b) Abolition of the slow response by adrenalectomy

Six rabbits were used in these experiments. In three animals the effect of stimulation of the hypothalamus on the seminal vesicle before and after bilateral extirpation of the adrenal glands was compared. One such experiment is illustrated in Text-fig. 2. In this case the quick response was abolished by section of the hypogastric nerve before adrenalectomy, leaving only the slow response of the vesicle to hypothalamic stimulation. After removal of the adrenals, further stimulation of the hypothalamus failed to produce any response. But injection of 5 μg adrenaline still induced a contraction of the vesicle, confirming that the operative interference had not markedly reduced the sensitivity of the organ. In a second rabbit this sequence was reversed.



Text-fig. 3. Effect of adrenalectomy on response of the seminal vesicle to hypothalamic stimulation. Right adrenal gland was removed 1 week before the experiment. Effect of stimulation of posterior hypothalamus (Hyp.) is shown before and after removal of left adrenal gland. Responses to injection of 2 and 5 μg adrenaline (2 A, 5 A) are also recorded. Time intervals 30 sec.

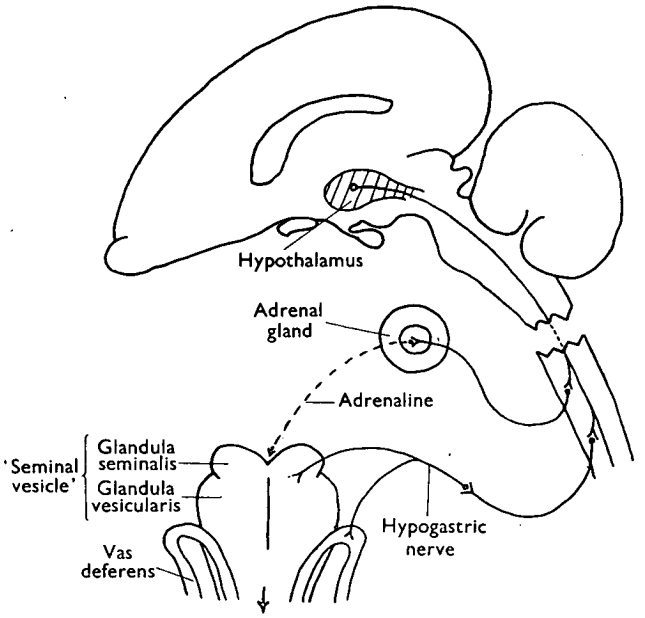
After bilateral adrenal cauterization only a quick response with a latency of 2 sec resulted from hypothalamic stimulation. This, too, was abolished by section of the hypogastric nerves. A third animal, however, continued to show a diphasic response of the vesicle after cauterization of the adrenals, but the size of the slow response was much reduced as compared with that obtained previously. When the adrenal sites were inspected in this animal at the end of the experiment, it was found that a large undamaged part of the right adrenal gland had remained adherent to the posterior vena cava.

Three other animals were prepared by a preliminary operation to remove the right adrenal only. About a week later stimulation of the hypothalamus was performed, and a diphasic response of the seminal vesicle recorded. Then the left adrenal was removed and stimulation of the hypothalamus repeated. In one animal a small delayed response to hypothalamic stimulation persisted after removal of the second adrenal. Subsequent *post-mortem* examination, however, revealed a large uncauterized

remnant of the right adrenal gland. In the other two animals there was no sign of a humoral response to stimulation of the hypothalamus when the remaining adrenal gland had been removed, although injections of small doses of adrenaline were still effective (Text-fig. 3). These experiments lend further support to the view that the slow response of the vesicle was due to secretion of adrenaline.

DISCUSSION

These experiments show that the hypothalamus can influence seminal emission in the rabbit. The route by which this influence is transmitted is a dual one, as illustrated in Text-fig. 4. Direct sympathetic pathways convey the excitation to the male organs, principally via the hypogastric nerves, and the effects so produced are then augmented by adrenaline released from the adrenal medulla.



Text-fig. 4

This evidence of a sympathetico-adrenal discharge resulting from stimulation of the hypothalmus in the rabbit is in good agreement with that previously obtained using the lactating mammary gland and oestrogenized uterus as indicator organs [Cross, 1955, 1957]. In all three cases the region of the hypothalamus from which sympathetico-adrenal effects could be elicited comprised the dorsal, lateral and posterior areas; the ventromedial area was always unresponsive. Another common finding concerns the amount of adrenomedullary hormone secreted following similar stimulation of the hypothalamus. The response of the seminal vesicle could be matched by i.v. injection of 1–5 μg adrenaline. The same dose was found to simulate the mammary and uterine effects of hypothalamic excitation in the earlier experiments. It is of interest that the sensitivity of all three test organs to adrenaline was about the same, the threshold dose in each case being approximately 1 μg. Moreover, the seminal

vesicle, mammary gland and uterus were all more responsive to adrenaline than to noradrenaline. An important distinction between the behaviour of the seminal vesicle and that of the other two organs, however, was failure of the vesicle to respond to physiological doses of oxytocin and vasopressin, or to stimulation of the neurohypophysis.

It is pertinent to ask if the present findings have a significance beyond the mere provision of a new test organ for studying sympathetico-adrenal activity. Is it possible that this hypothalamic mechanism plays some part in the control of accessory reproductive organs? Marshall [1922] cited evidence that erection and possibly ejaculation may result from stimuli from the brain, but very little work has been done on seminal emission. It seems generally accepted that emission and ejaculation of semen are events dependent on spinal reflex mechanisms. Thus Bacq [1931] obtained erection and ejaculation in rats after section of the spinal cord between the last thoracic and first lumbar vertebrae. Dusser de Barenne & Koskoff [1932] found that decapitation in cats produced erection, and reflex ejaculation could then be elicited by dorsiflexion of the tail. Applying direct electrical stimuli to the spinal cord in sheep and goats Gunn [1936] obtained seminal emission at lumbar levels and ejaculation at sacral levels.

It seems likely, however, that in the intact, conscious animal higher nervous centres participate in the control of seminal discharge. Experience with artificial insemination techniques in cattle breeding has shown that the degree of sexual excitation is an important factor governing the volume and quality of the semen ejaculates [Milovanov & Smirnov-Ugrumov, 1940]. A recent study by Kerruish [1955] has demonstrated, moreover, that an intensive regimen of precoital sexual stimulation in ten bulls raised the conception rate achieved by insemination of their ejaculates by 8·7 %. Evidently the improved ejaculates obtained in conditions of sexual excitement result from the greater efficiency of the contractile mechanisms in seminal emission and ejaculation. The present work suggests that a sympathetico-adrenal discharge from the hypothalamus might contribute to the increased discharge of semen by augmenting the contractions of the accessory organs. Other possibilities would be a central facilitation of spinal reflex excitability, and an enhanced sensory discharge from penile receptors in maximal erection, as suggested by Kitchell, Campbell, Quilliam & Larson [1955]. Further experimental work is required to define more precisely the central nervous mechanisms involved in these phenomena.

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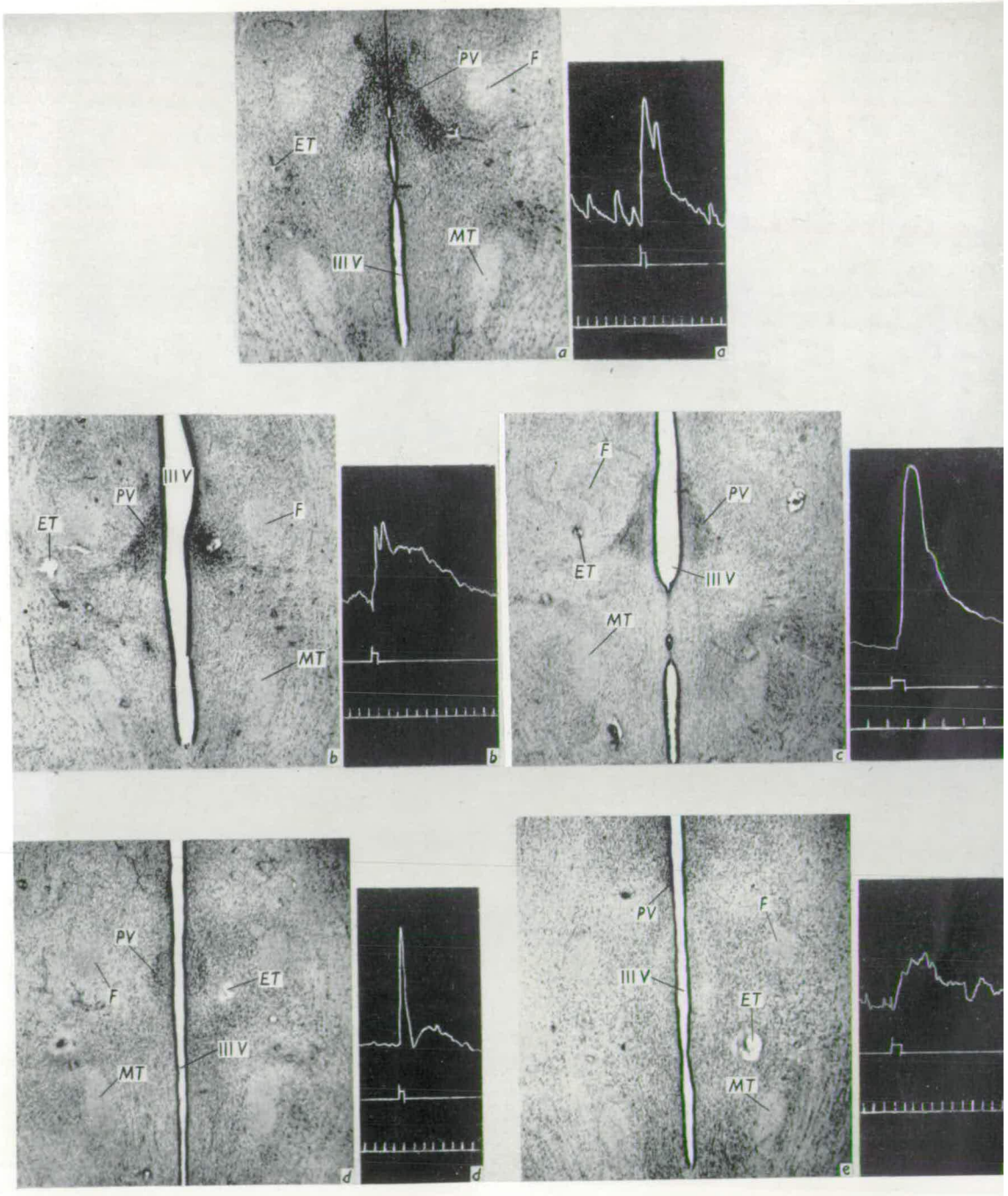
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DESCRIPTION OF PLATE

Kymographic records of typical responses of seminal vesicle to stimulation of the hypothalamus in five rabbits. The macrophotographs (× 15) on the left of each record show the relevant site of stimulation. The frozen sections were cut horizontally at 100 μ and stained with thionin.

Figs. *a–c* illustrate experiments on intact rabbits, and Figs. *d* and *e* experiments on unilaterally-adrenalectomized rabbits. *ET*, site of tip of stimulating electrode; *F*, fornix; *MT*, mammillothalamic tract; *PV*, paraventricular nucleus; III *V*, 3rd ventricle. Time intervals of kymographic records in 30 sec.

Temperature and epididymal function.

EXPERIMENTAL INDUCTION OF SEMINAL DEGENERATION IN RABBITS

by T. D. GLOVER, M.A., PH.D., B.SC., M.R.C.V.S.

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VIII

EXPERIMENTAL INDUCTION OF SEMINAL DEGENERATION IN RABBITS

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The fact that the testes of many mammals function at a temperatur which is lower than that of the abdomen is well recognized, and Moor and Quick (1923, 1924) established the vital role of the scrotum i regulating the temperature of the testes. This temperature together wit that of the epididymis may be increased experimentally to equal th temperature of the abdominal cavity by means of artificial cryptorchi dism in laboratory animals or insulation of the scrotum in larg animals such as bulls or rams.

In a previous communication (Glover, 1955b) it was shown tha insulation of the scrotum in rams evoked distinctive changes in th morphology of ejaculated spermatozoa, and this was in good agreemen with the findings of others referred to in that paper. With regard t laboratory animals, however, although the influence of experimenta cryptorchidism on the activity of ejaculated spermatozoa has bee recorded (Heller, 1929) there has hitherto been no clear informatio concerning its effect on morphology, in conditions of regular ejaculation Phillips and McKenzie (1934) claimed that in guinea-pigs the highe temperature of the abdomen exerted no pronounced influence on th morphology of spermatozoa in the epididymis; if this finding als applied to rabbits it would suggest that the early development o sterility resulting from cryptorchidism (Knaus, 1932) might be due t some factor other than morphological abnormality in the spermatozoa It seemed, therefore, that investigation of the effect of experimenta cryptorchidism on the morphology of ejaculated spermatozoa would b valuable, and the present paper reports on these effects in adult rabbits

The relative ease with which whole ejaculates can be obtained fro rabbits should afford an opportunity for a controlled experiment i which ejaculated spermatozoa rather than those in the epididymis ca be studied together with any special features of the semen that migh be occasioned by regular ejaculation. Rats and guinea-pigs do not offe this facility, because their semen cannot be collected so conveniently a regular intervals. Accordingly, rabbits were chosen as experimenta

animals for the present work, and specific tests for semen characteristics were applied. One purpose of the work was to determine whether or not these tests might be used to advantage in investigations of reproductive failure in male rabbits. Reciprocally, experimental cryptorchidism was tested as a method of inducing seminal degeneration.

MATERIAL AND METHODS

Observations have been made on sixteen adult male rabbits of mixed strain; they were kept under the same conditions of general environment and diet (compound pellets, Diet 18, Oxo, Ltd.). Permanent access to drinking water was provided for each animal.

Semen was collected from the rabbits by means of an artificial vagina (Macirone and Walton, 1938) and a minimum of three samples was taken from each animal before any operative interference. Each rabbit was then subjected to experimental cryptorchidism and two days allowed to elapse before further semen samples were taken. Throughout the experiment semen was collected at approximately three-day intervals.

Examination of semen

Semen was collected in graduated glass tubes so that the volume of each ejaculate could be measured directly. A homogeneous mass of gelatinous material was contained in most, but by no means all, ejaculates. If present, this 'gel' was removed with forceps before the semen was examined microscopically. A motility score from 0-5 was given to each semen sample from visual examination under the microscope, and for estimates of the percentage of dead spermatozoa in a sample, 100 spermatozoa were counted on semen smears stained with aqueous nigrosin eosin. These procedures were carried out with all materials and equipment being kept at 30° C., and in addition, the collecting tubes were warmed slightly before collection of the semen to avoid undue temperature shock (see Hancock, 1951 and Beatty, 1957). Morphological features of the spermatozoa were examined as previously described for ram semen (Glover, 1955b).

It is to be noted that in this work, all spermatozoa which were eosinophilic have been regarded as dead, and no detailed classification of the type described by Beatty (1957) has been adopted.

The total concentration of spermatozoa was estimated by the method of Walton (1927) on Fuchs-Rosenthal cytometer slides after dilution of whole semen to 1:200 with formalin saline.

Operative procedure

Each rabbit was anaesthetized with intravenous sodium pentobarbitone (Veterinary Nembutal solution, Abbott Laboratories), the urinary

bladder was emptied manually and the abdomen shaved before th operation which was performed with observation of aseptic precaution A midline laparotomy incision was made and extended almost to th pubis. The urinary bladder was displaced to one side and the test delivered into the abdominal cavity by gentle traction. Further tractio

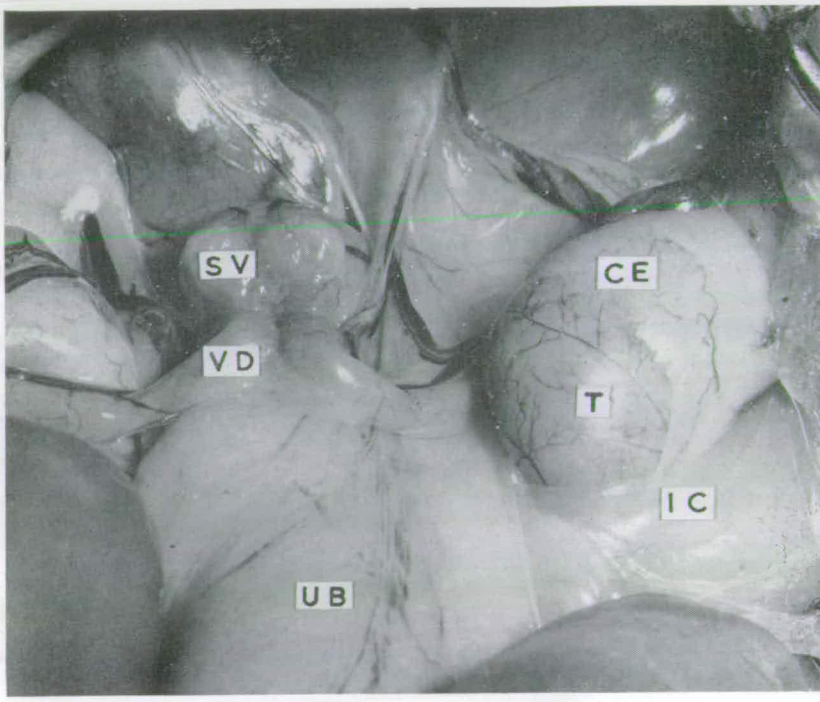

FIG. 1. Route by which testis is brought into abdominal cavity.

V.D. = Vas Deferens
T. = Testis
C.E. = Caput Epididymidis
I.C. = Inguinal Canal
U.B. = Urinary Bladder
S.V. = Seminal Vesicle (Glandula Vesicularis and Glandulae Seminales)

on the testis itself brought the gubernaculum testis into view (see Figs 1 and 2). The gubernaculum testis or inverted tunica vaginalis was the attached to the abdominal musculature by a single suture as shown i Fig. 2. In this way, the testis was secured in the abdomen and prevente from returning spontaneously into the scrotum. Furthermore, an trauma that might have been incurred by a suture through the testi itself was avoided. This procedure was repeated with the contralatera

testis. The muscle layers were closed with a continuous mattress suture and the skin with interrupted nylon sutures. Examinations *post mortem* showed both testes to have maintained a relatively constant position in the abdomen for periods of up to 30 days, and therefore any additional sutures for securing the testes were regarded as unnecessary.

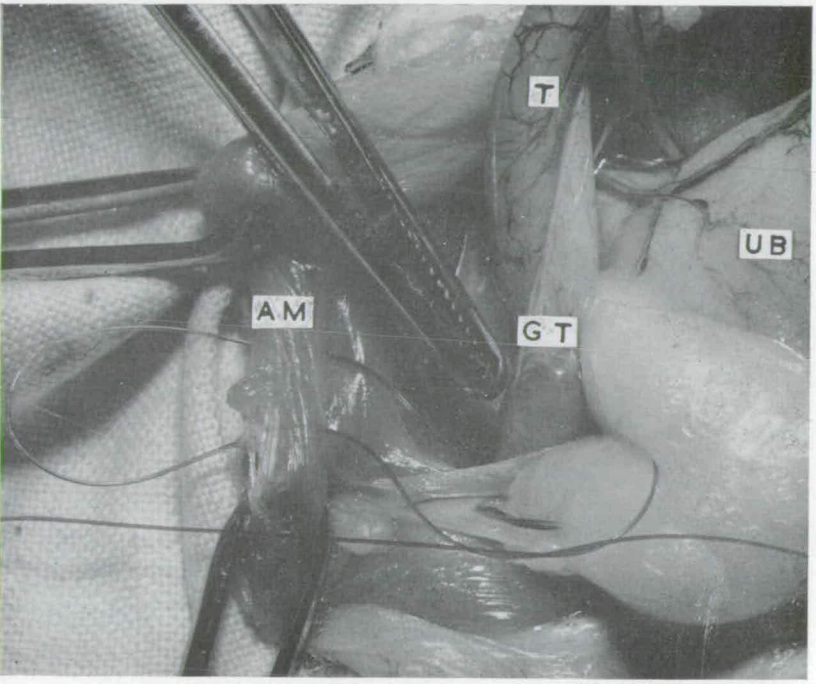

FIG. 2. Method of securing testis in the abdomen (see text). The forceps in the centre of the field are pointing at the inverted tunica vaginalis.

T. = Testis
G.T. = Gubernaculum Testis
U.B. = Urinary Bladder
A.M. = Abdominal Musculature

At the end of the experiment, viz., when the semen of each animal was virtually devoid of spermatozoa, the rabbits were destroyed and the testes and epididymides examined histologically. Segments of both structures were cut with a razor, fixed in aqueous Bouin's solution and sections were cut at 7μ. The sections were stained with Weigert's haematoxylin and eosin.

FIG. 3. Photomicrograph showing typical picture of incipient seminal degeneratio (stained with aqueous nigrosin eosin) (×355).

FIG. 4. Photomicrograph showing typical picture of more advanced semin degeneration (stained with aqueous nigrosin eosin) (×355).

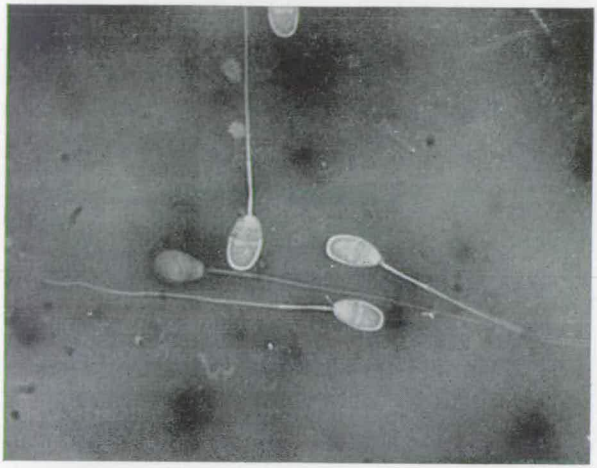

FIG. 5. Normal rabbit spermatozoa stained with aqueous nigrosin eosin (×990).

FIG. 6. Coiled tailed spermatozoa (×990).

FIG. 7. Decapitated spermatozoa (×990).

FIG. 8. Decapitated spermatozoa with detached tail (and middle piece) (×990
(see text).

RESULTS

Throughout these experiments no difficulty was experienced in persuading rabbits to copulate. Recovery from the operation was uneventful and all animals could be relied upon to produce a semen sample 72 hours after their operation. Before the main experiment was performed, seminal degeneration following experimental cryptorchidism

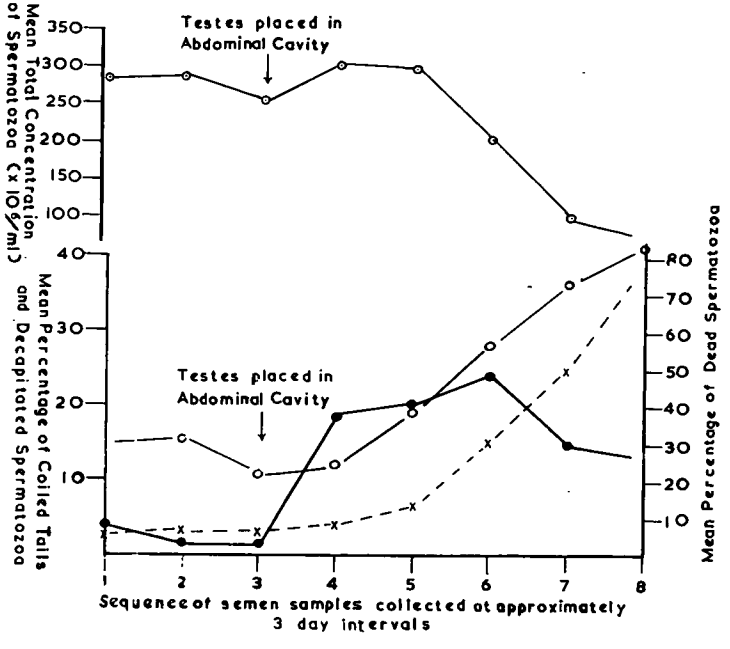

FIG. 9. Semen test characteristics of the rabbits before and during experimental cryptorchidism.
Mean values are given from data obtained in eight rabbits.
⊙———⊙ Mean concentration of spermatozoa $\times 10^6$/ml. O———O Mean percentage of dead spermatozoa.
× — — — — — × Mean percentage of decapitated spermatozoa ●———● Mean percentage of spermatozoa with coiled tails.

was observed in eight rabbits; of these, three were in a preliminary trial and five were part of another experiment that involved the experimental production of cryptorchidism. The pattern of degeneration in semen samples was characterized in each case by the appearance in ejaculates of an increased number of spermatozoa with coiled tails preceding a high proportion of decapitated spermatozoa. Typical examples of these

changes are shown in Figs. 3 and 4. Oligozoospermia was a feature of the process of degeneration. In the main experiment, a further eight rabbits were subjected to experimental cryptorchidism, and in order to follow its effects on semen quality more closely, quantitative estimates of different semen characteristics were made.

An increased proportion of spermatozoa with coiled tails was again indicative of the onset of seminal degeneration. Most of these forms of spermatozoa were non-eosinophilic (compare Figs. 5 and 6), though eosinophilic examples became more evident as degeneration proceeded. Decapitated spermatozoa (see Figs. 7 and 8) which followed in subsequent semen samples were to be found throughout the period of seminal degeneration. A gradual decline in the total concentration of spermatozoa was again apparent, but the actual onset of aspermia gave the impression of developing rather suddenly after periods of from 20 to 30 days.[1] Estimates of the total concentration of spermatozoa were rather variable in different rabbits, and for this reason the data were included in the statistical analysis.

Results from estimates of the total proportion of dead spermatozoa, the percentage of spermatozoa with coiled tails and of decapitated spermatozoa, as well as the total concentration of spermatozoa were analysed by using the analysis of variance technique. Classification into the baseline period (period before the operation) and treatment period (period after the operation) was made, with the sources of variation being between periods and between rabbits; the interaction between these was also tested. The mean squares and estimated values of F (see Fisher, 1948) are given in Table 1. The table shows that variation between periods was highly significant for morphological characteristics and the percentage of dead spermatozoa, though variation in total concentration was only significant at the 5% level (tabulated value of F at the 5% level of significance for 1.48 degrees of freedom = 4.0).

Variation between rabbits was also found to be significant at the 5% level (tabulated value of F at the 5% level of significance for 7.48 degrees of freedom = 2.2) with regard to the percentage of dead spermatozoa, more significant (1% level) for spermatozoa with coiled tails and highly significant for the total concentration of spermatozoa. No significant variation between rabbits was evident as far as decapitated spermatozoa were concerned.

These results mean that although, with the exception of decapitated spermatozoa, variation existed between estimates in the semen of different rabbits, the difference between all estimates in the baseline period and those in the treatment period was significant in every case (the concentration of spermatozoa diminishing, while other estimates

[1] Animals were termed 'aspermic' when no spermatozoa could be observed using the technique of semen examination described in the text.

ncreased). Indeed, this difference was highly significant for all criteria except the concentration of spermatozoa. There was no significant nteraction between the two sources of variation except in respect of spermatozoa with coiled tails.

Semen characteristics at different stages of the experiment are represented by mean values in Fig. 9. Owing to the fact that semen

TABLE I

ANALYSIS OF VARIANCE OF SEMEN CHARACTERISTICS

Mean Squares (M.S.) and Ratio of Mean Squares (F)

Source	*Degrees of freedom*	*% dead spermatozoa*		*% sperm-atozoa with coiled tails*		*% decapitated spermatozoa*		*Total concentration of spermatozoa (× 10⁶/ml.)*	
		M.S.	*F.*	*M.S.*	*F.*	*M.S.*	*F.*	*M.S.*	*F.*
Between Periods	1	11957	23.3***	3569	54.9***	3485	16.8***	109975	5.8 *
Between Rabbits	7	1151	2.2*	282	4.3**	101	0.5	165831	8.7***
nteraction	7	526	1.0	250	2.3*	64	0.3	31460	1.7
Residual	48	513		65		207		19057	
Total	63								

Levels of significance are represented as follows:

5% level of significance (*)
1% level of significance (**)
0.1% level of significance (***)

samples were not all collected on the same days after the operations, the values are only accurate to within three days. For example, a distinct increase in the percentage of spermatozoa with coiled tails shown to occur in the fourth sample (i.e. on the third day after the operation) is to be interpreted as meaning that the event took place in the semen of different rabbits between three and five days after the operation. Similarly, marked decapitation occurred 9-11 days after cryptorchidism had been produced (i.e. the third post-operative sample in Fig. 9).

The percentage of dead spermatozoa as estimated by the nigrosin eosin method was not influenced in the incipient stages of degeneration in the semen samples, although many spermatozoa with coiled tails had already made their appearance. A profound increase in the total proportion of dead spermatozoa was more closely associated with the onset of the decapitation phase as shown in Fig. 9. This was not entirely due to the percentage of dead sperm heads, however, because

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there was at this time an overall increase in the proportion of stained spermatozoa irrespective of their morphology.

Motility of semen declined as degeneration developed, but minor changes that might have been incurred would be obscured in this experiment owing to large variations in motility both between and within individual rabbits. Additionally, the method of scoring was rather arbitrary, but certainly complete cessation of motility was not apparent until the terminal stages of seminal degeneration when the few remaining spermatozoa were all dead. Thus, although a close relationship was obvious between motility and the percentage of dead decapitated spermatozoa, a high proportion of these forms in semen samples did not at first mean complete loss of motility.

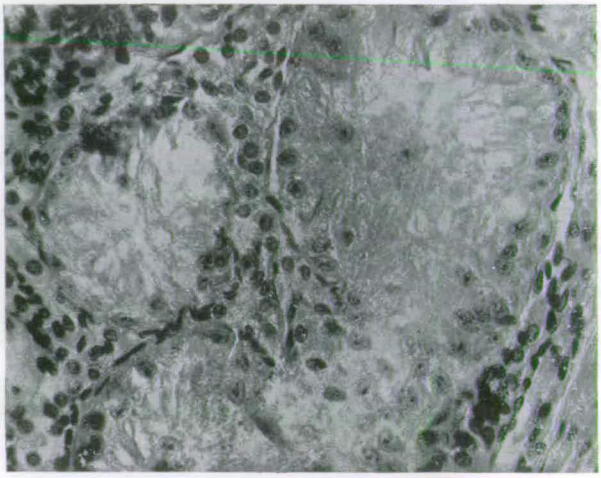

FIG. 10. Photomicrograph showing inhibition of spermatogenic activity due to 2 days of experimental cryptorchidism (× 150).

A further observation associated with decapitated spermatozoa is worthy of mention. As shown in Fig. 8 many loose dead sperm heads in the phase of decapitation were accompanied in the semen by tails (with middle pieces). This indicates the likelihood of separation of heads occurring in the fully formed spermatozoa rather than being a spermiogenic defect.

The volume of ejaculates varied considerably both within and between rabbits throughout the experiment, and the presence or absence of 'gel' was undoubtedly an underlying factor, but even when this was removed and the variability thereby reduced to some extent, no effect of cryptorchidism on semen volume was apparent.

When the rabbits were sacrificed after periods varying from 20 to 30 days of cryptorchidism, the testes were seen on examination to be unquestionably reduced in size. Histological examination showed typical destruction of spermatogenic cells so that only Sertoli cells and perhaps a few spermatogonia were evident at the periphery of the seminiferous tubules (see Fig. 10). The tubules of the epididymides contained no spermatozoa of any form in appreciable numbers.

DISCUSSION

The appraisal of semen is a well-established procedure in the assessment of fertility, not only because semen characteristics suggest the fertilizing capacity of any particular ejaculate, but also because they are a partial reflection of functional status within the male organs of reproduction. On these grounds, the manner in which abnormality of function within the male genital tract is revealed in the semen, becomes of considerable interest.

This work has shown the effects of experimental cryptorchidism in rabbits to be characteristically revealed in ejaculated semen. Furthermore, the induced abnormalities in the morphology of spermatozoa appear to be almost a facsimile of those resulting from insulation of the scrotum in large animals. While analogous changes in the histology of the testes have been previously shown to result from both these experimental procedures (Moore and Oslund, 1923, 1924), similarity in the pattern of seminal degeneration has not hitherto been recorded.

A feature of the semen following both cryptorchidism in rabbits and insulation of the scrotum of large animals is the non-eosinophilic character of spermatozoa with coiled tails in the incipient stage of degeneration. Moreover, in both these conditions there is some delay before abnormal spermatozoa first make their appearance in ejaculates. The length of this delay seems to vary in different circumstances. Walton and Uruski (1946) found that when rabbits were exposed to low atmospheric pressures, abnormal spermatozoa did not appear in ejaculates for about a fortnight. These workers concluded that the source of damage to the spermatozoa was in this case in the testes, and the time lag was entirely due to migration down the epididymis. A delay, varying from 5 to 13 days, has been recorded for decapitated spermatozoa to appear in the semen following prolonged insulation of the scrotum in rams, yet 17 to 24 days elapsed before the same effect was apparent after a short period of insulation of the scrotum (Glover, 1955a, 1955b). In the present work, coiled tails first became apparent in the semen 3 to 5 days after cryptorchidism had been produced and decapitated spermatozoa were observed after 9 to 11 days. The origin of these abnormal forms thus still remains conjectural, but it is interesting to speculate upon the relative resistance of spermatozoa to increased

temperature at different levels of the epididymis in rabbits. Experiment on this problem are now in progress.

Another factor associated with the time of onset of seminal degenera tion is the frequency of ejaculation. Young (1931) pointed out th importance of the extent to which copulation influenced sperm migra tion, but the relationship still remains uncertain. Frequency of ejacula tion has been reported to have little effect on the time of appearance o abnormalities in the semen following scrotal insulation in rams (Glover 1956b) but this finding remains to be confirmed in the semen of rabbit following experimental cryptorchidism.

Although several papers (Fukui, 1923; Cunningham, 1927; Asdel and Salisbury, 1941) have described the histological changes of the testes brought about by experimental cryptorchidism in rabbits, Knaus (1932) went further by demonstrating the manifestation of these changes in terms of fertilizing capacity. By fertility tests and examinations of semen it was shown that though the ability of semen samples to fertilize was lost after only four days of experimental cryptorchidism, sperma-tozoa in the same conditions did not lose their power of movement for twelve days. This result, while conflicting to some extent with the find-ings of Young (1927) in guinea-pigs and Elfving (1950) in rats (where the development of sterility was much more delayed), compares well with the result of the present experiment where the motility score and the per-centage of dead spermatozoa were not affected after three to five days of cryptorchidism. Nevertheless, it may be noted that a high proportion of spermatozoa with coiled tails occurred in ejaculates at this time. Could it be, therefore, that this abnormality participates in or even accounts for a loss of fertility, but at the same time exerts no influence on the motility score? Presumably, decapitated spermatozoa are incapable of fertiliza-tion, and in any case they are accompanied by an overall increase in the percentage of dead spermatozoa, but the significance of spermatozoa with coiled tails is more equivocal and warrants further study.

At all events, estimates of morphological characteristics, the per-centage of dead spermatozoa and their relation to fertility, would appear to be important. In contrast, considerable variation in estimates of semen volume, motility and total concentration of spermatozoa, even in untreated animals, suggests that these tests might not be as reliable.

The effect of elevated temperature on the hormonal activity of the testes is a controversial problem, but in large animals no loss of libido has been reported following insulation of the scrotum (Glover, 1956a), and there is evidence that such treatment might give rise to an increase in male-sex hormone activity (Meschaks, 1953). It may only be added here that the libido of rabbits did not appear to be in any way dimi-nished by cryptorchidism.

In the present experiment aqueous nigrosin eosin has been used for staining spermatozoa. The results illustrate the convenience of this stain for obtaining estimates of several different semen characteristics. Since Chang (1943) and Walton and Uruski (1946) used the method of semen examination in rabbits in studying the respective effects of low temperature and atmospheric pressures on the production of spermatozoa, it is conceivable that the semen tests employed in the present work for an investigation of cryptorchidism could be equally well applied to studies of other factors which might influence male fertility.

In conclusion, it may also be stated that experimental cryptorchidism has proved to be one useful method of inducing characteristic seminal degeneration in rabbits. The work is therefore a prelude to further study of this and other factors influencing male fertility.

SUMMARY

(1) An experiment is described in which some effects of experimental cryptorchidism on semen quality in rabbits have been investigated.

(2) Degeneration of semen induced by anchoring the testes and epididymides in the abdomen, was characterized by progressive oligozoospermia and distinct changes in the morphology of spermatozoa. Sexual drive and the total volume of ejaculates did not appear to be affected.

(3) Incipient seminal degeneration was marked by definite morphological features in the spermatozoa, despite there being no apparent effect on the incidence of active spermatozoa.

(4) Attention is drawn to the similarity of these results with those obtained following insulation of the scrotum in large animals, and the relative value of the different semen tests employed is discussed.

ACKNOWLEDGMENTS

The work was aided by a grant from the Agricultural Research Council. My thanks are due to Mr. D. Thurlbourn of Cambridge and Mr. W. J. McCabe of Liverpool for technical assistance and their care of the animals. I am indebted to Dr. A. M. Rajput of the University of Liverpool for help with the statistical analysis of results, and to Mr. W. Lee for the photography.

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Experimental cryptorchidism in rabbits: the response of spermatozoa in the tail of the epididymis. By T. D. Glover. *Department of Veterinary Anatomy, University of Liverpool*

Previous results have shown that decapitated spermatozoa appear in the ejaculates of rabbits after 9–11 days of experimental cryptorchidism [Glover, 1958]. Although several authors have speculated upon the origin of decapitated spermatozoa under conditions of increased temperature [see Phillips & McKenzie, 1934; Gunn, 1936; McKenzie & Berliner, 1937; Glover, 1955], there has been no direct evidence to indicate whether the defect occurs within the epididymis or in the testis. If decapitation in experimental cryptorchidism takes place entirely in the testes, it must be assumed that the morphology of spermatozoa already in the epididymis is unaffected by increased temperature. The present experiment was an attempt to test this assumption.

In ten rabbits the distal portion of the epididymis was isolated by means of ligation and removal of a small piece of the tubule proximal to the cauda epididymidis. This could be performed without significant interference with the blood supply. In each animal the cauda epididymidis on one side was isolated after delivery into the abdominal cavity, and together with the corresponding testis was secured to the abdominal wall by means of a suture passed through the inverted tunica vaginalis [see also Glover, 1958]. As a control measure isolation of the contralateral cauda epididymidis was carried out through the scrotum.

Three of the rabbits were killed at 3, 5 and 7 days after the operation, while the remainder were destroyed at random intervals up to 21 days. Spermatozoa from the isolated tail of the epididymis on either side of each animal were examined from smears stained with aqueous nigrosin eosin. In every case spermatozoa withdrawn from the tail of the epididymis situated in the scrotum, showed no abnormality in morphology that might not be observed under normal circumstances. Furthermore, no significant variation in the percentage of dead cells was apparent. Smears made from epididymides in the abdomen, however, showed a high incidence of both dead and decapitated spermatozoa in those rabbits destroyed after a period of 9 days.

The *maximum* difference in values obtained for the proportion of dead spermatozoa between the experimental (abdominal) and control (scrotal) epididymides was 9 % up to a period of 9 days. The same estimated difference for decapitated spermatozoa was 8 %. In the period between 9 and 21 days, however, the *minimum* differences were 40 % and 11 % for dead and decapitated spermatozoa respectively. The highest incidence of decapitated spermatozoa recorded in any one sample was 62 %; this estimate was made from a smear of spermatozoa withdrawn from an isolated distal tubule after 19 days of retention in the abdomen.

The results show that a great proportion of spermatozoa in the distal part of the epididymis of rabbits are susceptible to increased temperature, that the effect on the

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morphology of some of them is characteristic, but the response is not immediate. It is therefore suggested that some of the ejaculated spermatozoa with separated heads which appear in the semen early during experimental cryptorchidism are likely to be produced within the tail of the epididymis due to a cumulative effect of increased temperature.

The work was aided by a grant from the Agricultural Research Council.

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Semen Quality and Male Reproductive Function

BY

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THE cause of sub-fertility in bulls and other male animals is frequently not detected from examinations of semen, and it would seem that current methods of semen evaluation are inadequate for assessing all cases of impaired fertility. Nevertheless, it must be emphasised that very substantial error can be incurred in the interpretation of semen quality. For example, it is tempting to incriminate odd features of ejaculates such as a number of sperm abnormalities or a low concentration of spermatozoa as the cause of poor fertility. In many cases such casual interpretations are misleading or incorrect, and indeed great care is demanded if a useful interpretation of semen quality is to be achieved.

The significance of certain semen characteristics in relation to fertilising capacity is a vital criterion for the clinician who is called upon to forecast the fertility of a male animal. When a diagnosis of infertility or sub-fertility is required, however, some indication of the source and nature of the disturbance is also desirable. It is thus of some interest to ponder upon the extent to which the quality of semen might provide such information. Consideration of some fundamental processes involved in semen formation would seem to be appropriate in investigations of this problem.

From only a cursory glance at the life history of spermatozoa it is evident that the cells are exposed to various dangers throughout their existence. Such potential hazards include, even in the normal animal, failure to be ejaculated, lethal conditions *in vitro*, an unfavourable environment within the female or the absence of a fertilisable ovum. In an abnormal animal, disturbed conditions in the testes or in the epididymides might be superimposed (see Fig. 1).

At ejaculation, spermatozoa are mixed with accessory secretions which constitute the seminal plasma. This fluid exhibits marked variations in quantity and composition both within and between different animals. It cannot therefore be ignored in a genuine enquiry into an animal's reproductive capacity.

These essentials present themselves to the seminologist as a foundation for more detailed study.

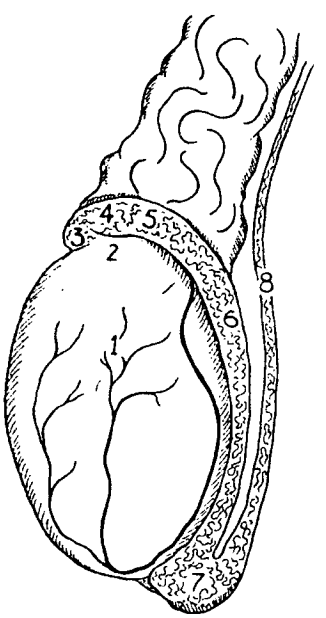


FIG. 1.—Testis of the bull showing the possible points at which spermatozoa might undergo damage. 1. Testis; 2. Vasa Efferentia; 3. Head of the epididymis proximal to the Caput flexure; 4. Caput flexure; 5. Head of the epididymis distal to the Caput flexure; 6. Body of the epididymis; 7. Tail of the epididymis; 8. Vas Deferens.

The Function of the Testes

It is not the province of this paper to attempt a comprehensive discourse on the physiology of the testes, and discussion will therefore be confined to those aspects of the subject regarded as being most germane to the problems of the clinician. Perhaps the most striking feature of the testes in the majority of domesticated mammals is their position in the scrotum outside the abdominal cavity. Despite an abundance of research on the subject, the precise reason for testes descending into a scrotum is a problem that remains incompletely solved.

As early as 1893, Griffiths observed testicular damage in dogs resulting from artificial cryptorchidism, but it was Crew (1922) who first concluded that the testicle might under normal circumstances require a lower temperature than existed in the abdomen. Moore (1923, 1924) produced degeneration in the testes of guinea-pigs by means of experimental cryptorchidism, Fukui (1923) induced similar changes in the testes of rabbits, and Moore and Quick (1923, 1924) conclusively illustrated the thermoregulatory function of the scrotum. The detrimental influence of increased temperature on spermatogenesis may now be regarded as being well established in other animals whose testes are situated in the scrotum. Moore and Oslund (1924) clearly demonstrated this in rams from studies using insulation of the scrotum as an experimental method, and Casady, Myers and Legates (1953) reported spermatogenic dysfunction in bulls following exposure of the animals to increased ambient temperatures.

The relationship between testicular response to high temperatures and semen evaluation was demonstrated by Lagerlöf (1934) who showed that insulation of the scrotum in bulls resulted in characteristic degeneration of semen quality. Similar results have been obtained with rams (Phillips & McKenzie, 1934; Gunn, 1936; Gunn, Sanders & Granger, 1942; Glover 1955b). McKenzie and Berliner (1937) reported the same type of changes in semen characteristics of rams due to an increase in environmental temperature.

The influence of environmental temperature on reproductive function becomes a particularly important consideration when animals are being exported from temperate to tropical climates, though in these instances, other factors such as light and stress are also to be taken into account. The effects of local temperature elevation, however, might have wider application, for they are worthy of note with regard to any inflammatory condition of the gonads (whether or not it is of an infective nature). Orchitis and epididymitis of bulls due to *Brucella abortus* and *Corynebacterium pyogenes* infections have been admirably described by Blom and Christensen (1947), and it is evident that an increase in testicular temperature would be involved in conditions of this kind, irrespective of general systemic disturbance. Pyrexia, however, does appear to be markedly detrimental to testicular function. For instance, Gunn *et al.* (1942) observed seminal degeneration in rams after the intra-peritoneal injection of pus from acute abscesses. Similar changes in the semen of rams were reported by these workers in cases of strike, whether the blowfly maggot was on the scrotum or elsewhere.

It therefore seems clear that elevation of temperature whether local or general exerts a profound deleterious effect on the testes, but it would be unfortunate to presume that all pathological conditions of these organs are due to temperature variation. Hormonal, nutritional and genetic factors are probably more frequently involved. Nevertheless, Olesen (1948) stated that both in man and animals, except in cases irradiation, testicular degeneration took the course of passing from the most highly differentiated cells to the least; he quoted Schinz and Slotopolsky's tabulation (1924) of desquamation of spermatids and spermatocytes in that order; this is also the pattern of degeneration shown in thermal sterility. It might be well to keep in mind, however, that the damaging effect of increased temperature may not act altogether directly on testis tissue.

Walton and Uruski (1946) showed that low atmospheric pressure had a detrimental effect upon the fertility of male rabbits. Slight changes produced in the testes as a result of the treatment were entirely reversible on returning the animals to normal atmospheric pressures. In this paper references were made to others who had noted a loss of fertility in animals in which anoxaemia was produced from the inhalation of carbon monoxide. The work assumes importance relevant to temperature effects on the testes from the belief by some workers (Moore, 1924, and mentioned by Chang, 1943) that heat degeneration might be caused by the production of anoxia in the testes. From this, the manner in which blood circulates through the testes would seem to be of interest.

Extensive investigations into arterial patterns of the male gonads in a great number of species have been made (Harrison, 1948, 1949; Harrison & Barclay, 1948; Harrison & Weiner, 1949) and not only as an exceptional vascularisation of the mammalian testes been demonstrated by this work, but a relationship shown to exist between the arterial design and the abdomino-testicular temperature difference.

Legends to Art Plates

PLATE I.—Arteriograph of the testis of a bull following injection of the internal spermatic artery with 20 per cent. Chlorbismol (M. & B.). Inset: Cross-section of a bull's testis showing the course of the end arteries. Distal view of the arterial arrangement in the tail of the epididymis.

PLATE II.—Arteriograph of the testis of a ram.

PLATE III.—Arteriograph of the testis of a ram with the epididymal arteries teased out.

PLATE IV.—Arteriograph of the testis of a boar. Inset: Cross-section of a boar's testis.

PLATE V.—The arterial supply of the testis of a domestic fowl after injection with 20 per cent. Chlorbismol (M. & B.). Inset: Several cross-sections of the testis of a fowl.

PLATE VI.—"Vinylite" (Bakelite, Ltd.) cast of the artery and veins in the testis of a ram, illustrating the vascular arrangement in the Pampiniform Plexus. (See text.)

PLATES VII AND VIII.—Nigrosin eosin smears of semen taken from two rams about three weeks after a 24-hour period of scrotal insulation (× 450). This picture was obtained in three or four ejaculates only, after which a normal semen picture was resumed. Should such animals be pronounced infertile? (See text.)

T. D. GLOVER—SEMEN QUALITY AND MALE REPRODUCTIVE FUNCTION

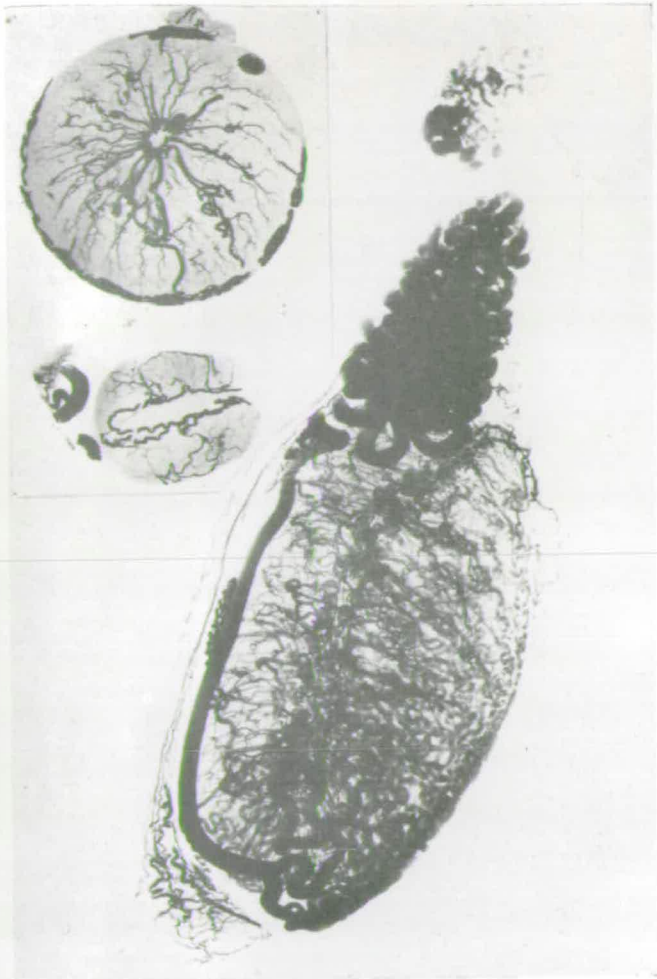
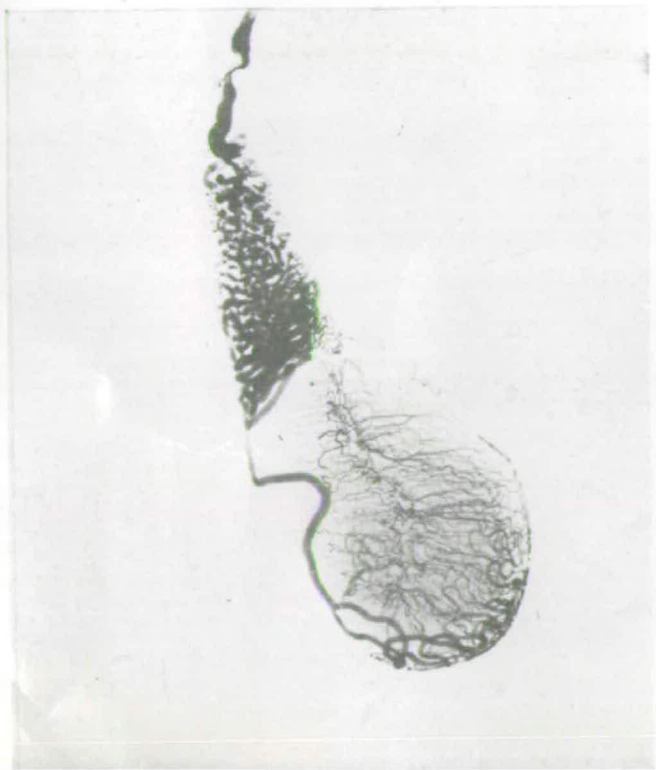


PLATE I.



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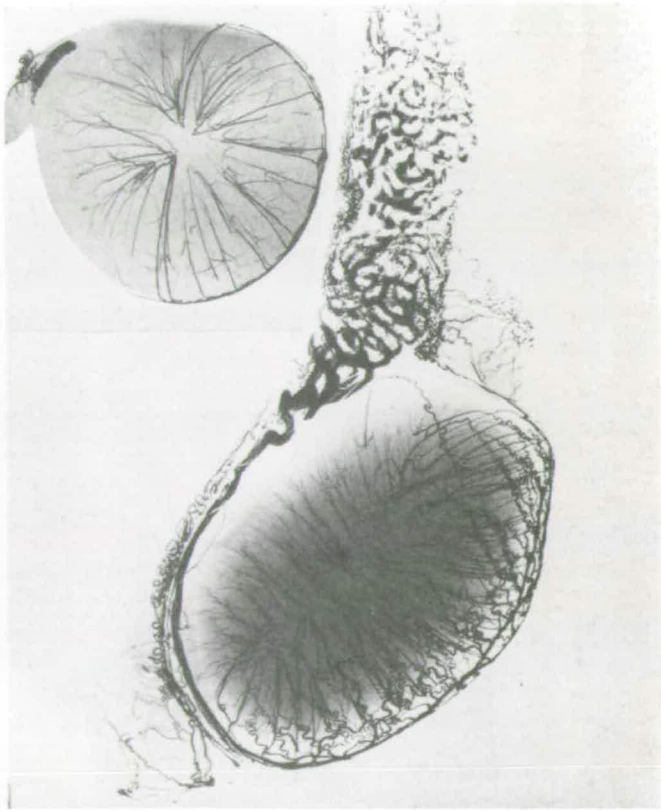

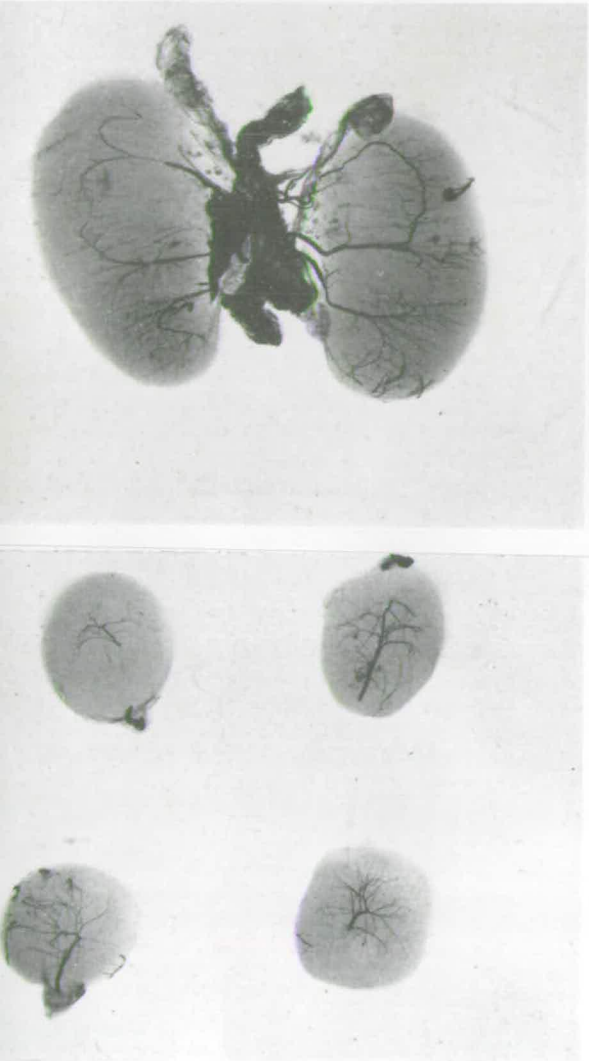

PLATE V

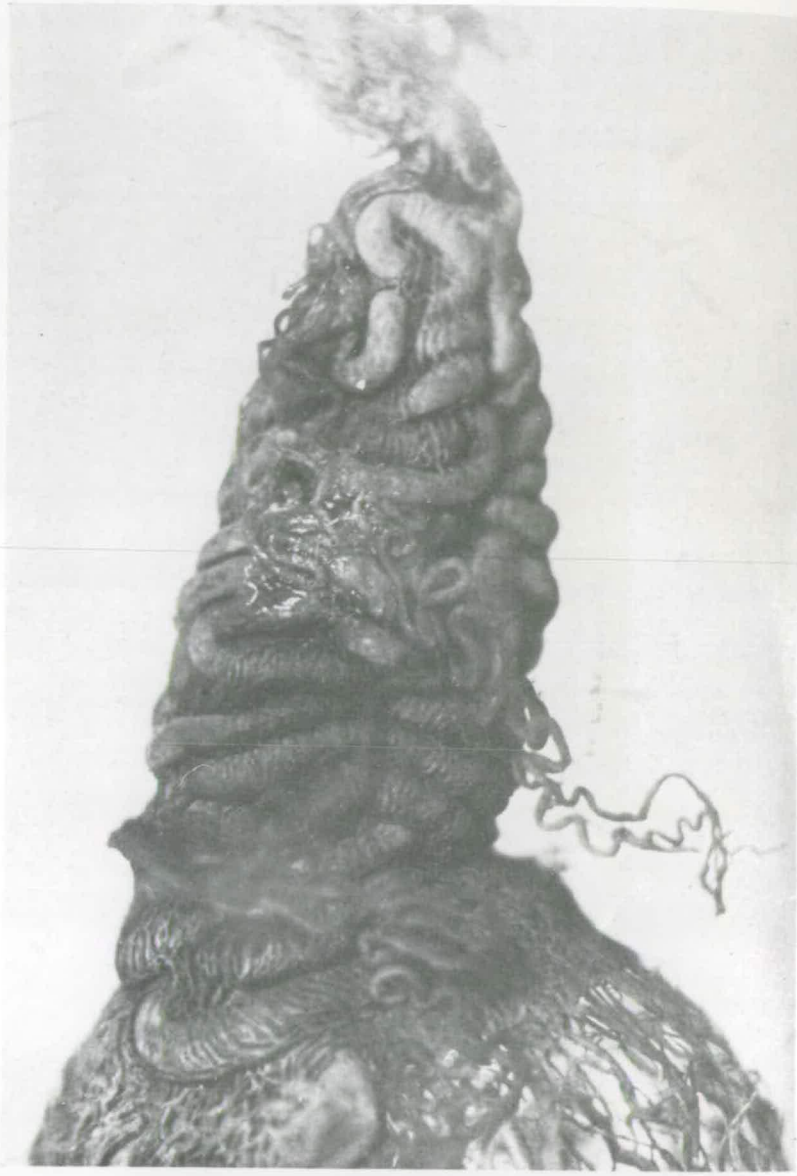

PLATE VI.

PLATE VII.

PLATE VIII.

Harrison (1948) showed that with the exception of n and the wallaby (Macropus) the internal sperm- (testicular) artery was variably convoluted before ching the testis, where it generally took a super- al course. Arteries entered the testis by means of tripetal branches coursing along the septa ; these nches then turned outwards to run centrifugally. detailed account of this type of vascular arrange- nt has been given with reference to the testis of bull (Kirby, 1953), and it is interesting to com- re this in some other species of domesticated imal (see Plates I to V). In the pig and ram the scular pattern may be described as typical whereas the fowl, where the testes are normally resident in abdominal cavity, the artery passes directly into testis parenchyma from the aorta. This suggests association between the unusual vascularisation testes and their descent into a scrotum, although dy of the testicular blood supply in mammalian sticonda would provide more direct information in is regard.

Surrounding the convoluted artery throughout ost of the spermatic cord is an extremely fine net- ork of veins draining blood from the testes (see ate VI). On the grounds that venous blood from e testes would be cooler than that flowing from the domen, Harrison (1948, 1949 & 1952) suggested at the pampiniform plexus served as a means of pre-cooling " the blood before it entered the testes. arrison and Weiner (1949) confirmed this sugges- on experimentally by means of thermo-couple easurements taken in the testicular artery. It is ot justifiable however, to argue that this could be e only physiological result of such anatomical atures ; a pronounced fall in blood pressure for stance, must also result from such a lengthy artery, nd the possibility of some gaseous or other chemical anges in the blood before it enters the testes cannot et be excluded. Coiling of the artery may also be garded as being convenient when it is considered at the testes move up and down in the scrotum eely, although such mechanical facilitation could ardly alone explain the excessive convolution in e artery.

In conclusion one must agree with Harrison and Veiner (1949) that more detailed study of the circu- tion of the testes is desirable. Measurements of xygen and carbon dioxide tensions in arterial and enous blood associated with the testes should also ield some interesting physiological information, and closer study of the phylogeny and ontogeny of the esticular blood supply might provide a valuable ontribution to our knowledge of the testes. From he clinician's point of view, however, it is evident hat whatever its function, the complicated arrange- ment of blood vessels in association with the testes vill render the organs extremely susceptible to disorders of circulation.

Damage within the testes may often be recognized n the semen by deformities of the sperm heads ndicating a disturbance of spermatogenesis. In dvanced degeneration a chronic oligospermia would e likely to accompany such abnormalities. It is quite evident, however, that abnormalities of sperm- tozoa may be incurred after spermateliosis and the passage of the cells into the efferent ductules. Blom (1950) classified abnormalities which he regarded as being of this type into " secondary abnormalities." From this it is clear that the function of the epididymis is a necessary consideration in any critical interpretation of semen quality.

The Importance of the Epididymis

Three main events characterise the sojourn of spermatozoa in the epididymal tubules. These are, migration, maturation and senescence. Spermatozoa undergo a process of maturation as they migrate down the epididymis so that they become mature within the tail of the epididymis and in the vas deferens. Here they wait, passively, for the sudden burst of energy that would be engendered by the relatively aerobic conditions or the addition of seminal fructose resulting from ejaculation (see Dott, 1958). The length of time spermatozoa take to migrate down the epididymis in different species is not yet exactly known but recent work with radioactive isotopes has opened a new avenue for investigating the problem (see Dawson, 1958). Gunn (1936) using electrical stimulation to obtain ejaculates, reported that this migration time in the ram was 5 to 6 days and 11 days in sexually rested animals. Phillips and McKenzie (1934) claimed a variation of 4 to 13 days for migration when allowing rams 3 services every 3 days. The frequency of ejaculation would certainly seem to be a factor that might effect the rate of migration, but the extent of its influence in this respect is not so far definitely established. Likewise the fate of spermatozoa in the sexually inactive animal is obscure, though evidence makes it clear that they are in some way ultimately destroyed and absorbed within the epididymis (Redenz & Belenoschkin, 1929 ; Young, 1931 ; Simeone & Young, 1931).

With regard to the maturation of spermatozoa in the epididymis, it is well known that one characteristic of the process is the migration of the cytoplasmic droplet from a position at the neck of the spermatozoon to the end of its middle piece. At ejaculation, the droplet is normally separated from the spermatozoon. Spermatozoa ejaculated with proximally situated droplets therefore, would seem to indicate defective function in the epididymis whereby the normal maturation of spermatozoa is inhibited. This is only one example of possible defect within the epididymis, but other cases may exist. It has not been proven for instance at what point decapitation of spermatozoa takes place in all circumstances. Hancock (1952) has shown that genetic decapitation in Guernsey bulls takes place in the epididymis in association with the migration of the cytoplasmic droplet. It has been suggested that heat induced decapitation can occur in the epididymis (Glover, 1955b, 1956) although Phillips and McKenzie (1934) believed that abnormalities of spermatozoa resulting from insulation of the scrotum were due to faulty spermatogenesis. McKenzie and Berliner (1937) nevertheless favoured the assumption that fully formed spermatozoa could be affected by a rise in temperature.

The time taken for decapitated spermatozoa to appear in ejaculates following a localised increase in temperature has presented some difficulty in interpretation for when the scrotum of bulls or rams is insulated, decapitated spermatozoa do not appear in ejaculates at once. This delay in appearance of decapitated sperms has also been shown to occur in rabbit ejaculates following experimental cryptorchidism (Glover, 1958). These results indicate that spermatozoa in the tail of the epididymis do not show an immediate adverse response in their morphology to increased temperature. Recent experiments on the isolated cauda epididymidis have shown this to be true as far as rabbit spermatozoa are concerned, because increased temperature appears to act cumulatively on sperms in the tail of the epididymis in this species (Glover, 1959). Nevertheless, the transitory appearance of decapitated spermatozoa can result from only 24 hours of insulation of the scrotum where a cumulative effect of heat would not be possible (Glover, 1955a). It is noteworthy, however, that the delay in appearance of decapitated sperms in these circumstances is longer than in prolonged treatment (Glover, 1955b). This suggests that in acute temperature elevation damage to spermatozoa might occur at a point nearer to the testes than in prolonged increased temperature. If a relatively small mass of spermatozoa can be decapitated at a point high in the epididymis, as short periods of scrotal insulation suggest, it would be of considerable clinical significance, because it would mean that a semen sample containing a very high percentage of decapitated spermatozoa could be produced by an animal whose testes were at that time functioning quite normally. In such circumstances subsequent ejaculates could be highly fertile when normal spermatozoa replace the mass of decapitated forms in the epididymis (see Plates VII & VIII). This shows that several ejaculates are demanded if an accurate assessment of reproductive activity is to be achieved.

The Value of Semen Tests

Several different tests have been described for the appraisal of semen samples, but after many experiments by different workers, the relative value of these tests on bull semen was assessed in an exhaustive investigation by Bishop, Campbell, Hancock and Walton (1954). The interrelationships of these tests have been succinctly presented in a form of value to the clinician by Bishop (1955) and Bishop and Hancock (1955). It is evident from this work that measurements of the physical activity of spermatozoa yield information which is as valuable as any and that for most clinical purposes complex metabolic tests serve little advantage. The difficulty of interpreting morphological abnormalities is nevertheless already apparent, and in addition, there is considerable interraction between different tests of even physical characteristics which may obscure a true result. For instance, it has been pointed out (Glover, 1955b) that acute scrotal dysfunction may bring about a short inhibition of impedance change frequency which is a method of assessing the motility of spermatozoa (see Rothschild, 1949), but a high proportion of dead and decapitated spermatozoa accompanies this effect. Thus there is interraction in this case between the percentage of dead spermatozoa, the proportion of decapitated spermatozoa and impedance change frequency. It is to be remembered, however, that a proportion of decapitated spermatozoa may not in itself necessarily effect the overall activity of semen samples, provided enough living spermatozoa are present. Thus a semen sample with many decapitated spermatozoa in it may exhibit more activity than another sample showing no morphological abnormalities. The influence of certain inherited abnormalities in sperm morphology upon fertility in bulls has been demonstrated (Hancock, 1949 & 1952), but the significance of such defects as coiled tails and in some cases decapitated forms still needs to be tested by insemination, for they might not always be of genetic origin.

If it is decided in any given case of seminal deficiency that genetic factors are not involved, the possibility of therapy might be considered. Unfortunately treatment with hormones has often proved disappointing and this would seem largely due to a paucity of precise information regarding the endocrine control of male reproductive activity. Indications for the clinical use of sex-hormones in male animals are thus at present very rare. Nevertheless there is evidence (Benoit, 1926; Moore, 1926; Moore & McGee, 1928; & Young, 1929, 1931) that the testes exert some influence upon the normal survival of spermatozoa in the epididymis, and Elfving (1950) has claimed that in rats, small doses of testosterone, thyroid hormone or F.S.H. can delay the onset of sterility resulting from the application of heat to the scrotum. It might be held therefore that hormonal activity will become a more important and practical consideration in clinical cases of male infertility.

The Significance of the Seminal Plasma

Until the physiological significance of seminal plasma has been more fully elucidated and the normal variation between individuals of any one species has been more completely assessed, detailed examination of the fluid portion of the semen remains primarily of specialist and experimental interest. In spite of this, an enormous increase in our knowledge of seminal plasma has taken place in the last few years, and the pioneer work of Mann and his coworkers has revealed some astonishing facts in this context that were hitherto completely unknown (Mann, 1954). The findings, for example, that male-sex hormone activity may be reflected in the level of seminal fructose (Mann & Parsons, 1947) and citric acid (Mann, Davies & Humphrey, 1949) serve alone to illustrate the potential value of constituents of the seminal plasma to the clinician. It may well be that in the future veterinarians will pay more detailed attention to the seminal plasma in assessing male reproductive function or the value of therapeutic agents. At present, estimates of the volume of fluid and visual examination of its consistency represent the clinician's approach, with more detailed laboratory tests being indicated in some cases.

It is important to emphasise that the volume of seminal plasma like the concentration of spermatozoa is partly a reflection of the power of emission and ejaculation, and it would seem that too little attention has been paid to the vital role which these reflex mechanisms play in the control of semen quality.

The Discharge of Semen

Kirillov and Morozov (1933) and Milovanov and Smirnov-Ugrumov (1940) have shown in bulls that the volume and sperm concentration of ejaculates is directly related to the degree of pre-coital sexual excitement, and the favourable influence of such nervous stimulation has been demonstrated recently by Kerruish (1955). Evidently the volume of semen represents not only the secretory activity of the accessory glands, but also the efficiency of their contractile mechanisms. It is also possible that fluctuations in the concentration or number of spermatozoa in semen may not always be related to sperm production by the testes. On these grounds, it is desirable to consider the chain of reactions leading to the discharge of semen at orgasm. These include erection of the penis, seminal emission and ejaculation. Since under certain circumstances each can occur independently of the other two, they may be regarded as separate physiological entities.

Erection. The bull, goat, ram and boar possess a fibro-elastic type of penis in contrast to the vascular penis of the stallion and dog, but in all these cases a degree of vasodilatation occurs in erection. It is generally agreed that erection is principally produced by parasympathetic activity *via* the pelvic nervi erigentes.

Emission. Seminal emission may be described as the passage of spermatozoa and seminal fluid into the pelvic urethra as a result of the contraction of smooth muscle in the accessory organs, such as the epididymis, vas deferens and seminal vesicles. This is under the motor control of the autonomic nervous system and chiefly seems to involve sympathetic outflow *via* the hypogastric nerves (Langley & Anderson, 1895).

Ejaculation. This is the process whereby semen is discharged from the urethra. In contrast to the other two reactions it involves contraction of striated muscle in the urethra and penis, which is innervated by somatic fibres in the pudendal nerves.

Little is yet known of the precise mechanisms which operate in the nervous control of reproductive processes in the male animal. Although it is certain that erection and ejaculation can occur in the spinal animal (Bacq, 1931 ; Dusser de Barenne & Koskoff, 1932 ; Spina, 1897) the manner in which the nervous system integrates these events is obscure. At orgasm, seminal emission and ejaculation occur in a co-ordinated sequence and it is not unreasonable to assume that stimuli from the brain may be involved. Erection certainly includes activity of the higher centres since it normally occurs before sexual contact is made. However, ejaculation may normally require an afferent input from the penis. Thus local anaesthesia of the penis (Cross & Glover, unpublished observations) did not prevent erection but completely inhibited ejaculation.

In 1922, Marshall suggested that ejaculation might include stimuli from the brain, but as with the work of Spina, which he quoted, no clear distinction was made between emission and ejaculation, and his conclusions are somewhat difficult to interpret. Recently, some indication of a central nervous mechanism presiding over emission has been provided from experiments with rabbits (Cross & Glover, 1958). This work showed that electrical stimulation of the sympathetic areas of the hypothalamus produces seminal emission, both *via* the hypogastric nerves and by means of the release of adrenaline from the adrenal medulla. Since it is known that a similar sympathetic mechanism can operate in other conditions of emotional arousal (Cross, 1955) it is quite possible that hypothalamic activity occurs in sexual excitement of the male animal giving rise to an improved quality of ejaculate.

Conclusions

Generally speaking a clinician is asked to examine a male animal for one of two reasons. Either a consultation is requested to ascertain whether an animal is capable of reproduction, or advice is sought when an individual's fertility is suspect. In either case, examination of semen and observations on sexual behaviour would seem to be the main approach for reaching an initial conclusion, provided a careful interpretation of the characteristics of ejaculates is made.

In an assessment of any semen picture it is to be remembered that deficient sexual function does not necessarily preclude an animal from reproducing its kind. On the other hand, while a completely sterile animal can be recognised by anyone who has the most superficial acquaintance with a normal semen picture, the origins of sub-fertility may be much more obscure, since often the semen picture may appear to be normal.

Despite these difficulties, emphasis has been placed in this paper on the recognition of abnormal reproductive function by means of the examination of ejaculates, for the quality of semen, which is after all the fertilising substance, should be the most helpful factor in arriving at a diagnosis or in giving a prognosis. This is particularly so since general physical deformity in a sire may be unrelated to fertility. If a diseased condition does exert an influence on fertility, then it would seem likely to be revealed in the semen or in copulatory behaviour.

On these grounds, general clinical examination of a male animal in an investigation of fertility would seem to serve as an accessory procedure to preliminary enquiry into semen quality and sexual behaviour. In spite of this, clinical examinations are called for in most cases of impaired fertility but caution must be observed in ascribing reproductive failure to remote diseased conditions, while remembering that they *may* in some cases participate.

Further research into the biology of mammalian semen should help to elucidate many problems and thus augment the value of semen examination as an aid to the clinician.

Acknowledgments.—I wish to thank Dr. B. A. Cross for his comments on this review. I am indebted to Mr. J. A. F. Fozzard of the Department of Anatomy, University of Cambridge for the arteriography, Messrs. Guntrip and Green of Cambridge for other photographs, and Mr. J. W. Dransfield of Liverpool for his drawing of Fig. 1.

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SPERMATOZOA FROM THE ISOLATED CAUDA EPIDIDYMIDIS OF RABBITS AND SOME EFFECTS OF ARTIFICIAL CRYPTORCHIDISM*

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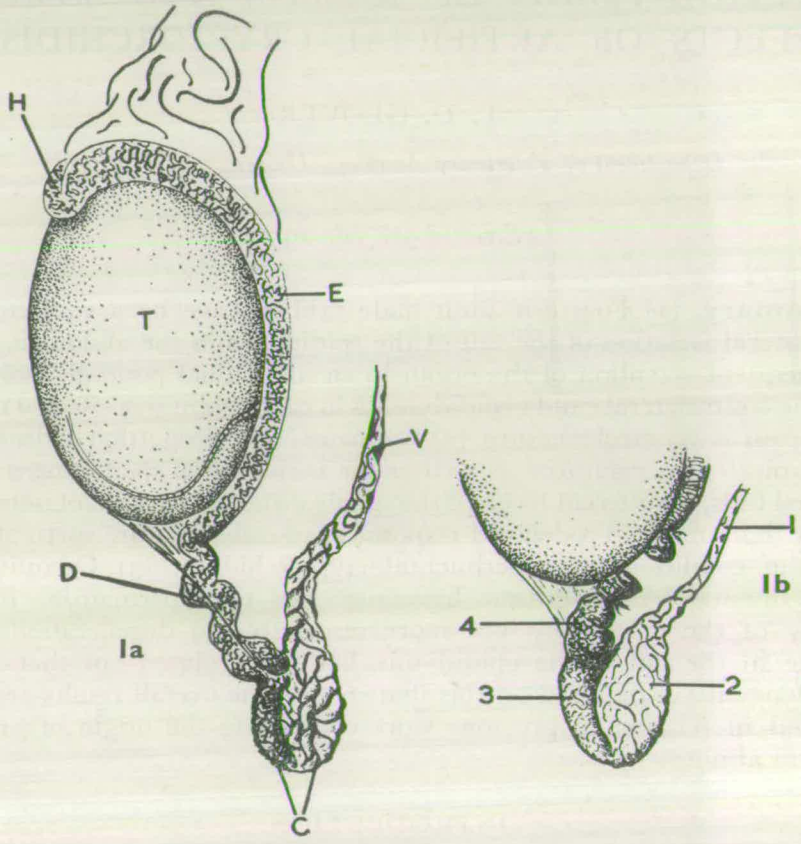
Summary. (1) Fourteen adult male rabbits have been subjected to unilateral isolation of the tail of the epididymis in the abdomen, with subsequent retention of the organ in an abdominal position. Isolation of the contralateral cauda epididymidis in each rabbit was effected in the scrotum as a control measure. (2) Estimates of the proportion of dead and decapitated spermatozoa as well as the incidence of spermatozoa with coiled tails, at different levels of the tubule distal to the point of isolation, have demonstrated a delayed response of so-called mature spermatozoa to the conditions of experimental cryptorchidism. (3) Quantitative assessments of these criteria have suggested that spermatozoa in the body of the epididymis are more susceptible to degeneration than those in the tail of the epididymis, but it is pointed out that more evidence is yet required on this matter. (4) The overall results are discussed in relation to previous work concerning the origin of similar sperm abnormalities.

INTRODUCTION

In an earlier paper (Glover, 1958), it was shown that an increased proportion of dead and decapitated spermatozoa were to be found in the ejaculated semen of rabbits after 9 to 11 days of artificial cryptorchidism. The origin of these degenerate spermatozoa was at that time in doubt, but more recent experiments involving ligated epididymides (Glover, 1959) have shown that many spermatozoa in the cauda epididymidis appear to die and become decapitated as a result of the conditions imposed by experimental cryptorchidism. A cumulative effect of increased temperature upon spermatozoa in this region has thus been postulated, but closer examination of the cauda epididymidis in rabbits discloses variation in the diameter of its tubule which detracts from it being regarded as a simple anatomical entity, despite the demonstration of a constancy in histological structure (Nicander, 1957). Furthermore, no clear evidence has hitherto been available to indicate whether spermatozoa throughout the cauda epididymidis react in the same way during artificial cryptorchidism.

* Read at the Annual Conference, 1959, of the Society for the Study of Fertility.

The present work reports on the characteristics of spermatozoa taken from different levels of the distal regions of the epididymis and from the vas deferens after their isolation from the more proximal parts of the tubule. By means of this more detailed scrutiny, an attempt has been made to discover the extent



TEXT-FIG. 1a. *A diagram of the testis and epididymis of the rabbit to illustrate the anatomical arrangement of the distal regions of the tubule.* Observe the spiral arrangement of the lower part of the corpus epididymidis around a fibrous cord (see text). T = testis. H = head of epididymis. E = body of epididymis. D = distal part of corpus epididymidis (isolated in this experiment together with the cauda epididymidis and vas deferens). C = cauda epididymidis. V = vas deferens.
TEXT-FIG. 1b. *A diagram to illustrate the method of isolation of the tail of the epididymis and levels of the tubule from which spermatozoa were withdrawn.* 1 = vas deferens. 2 = widest part of cauda epididymidis. 3 = proximal region of cauda epididymidis. 4 = distal part of corpus epididymidis.

to which morphological characteristics of spermatozoa taken from any one level of the distal tubule are representative of spermatozoa in the cauda epididymidis as a whole.

ANATOMICAL CONSIDERATIONS

The epididymis of the rabbit is typical of that of many other mammals in that it appears to have a head, a body and a tail. The narrow tubule of the

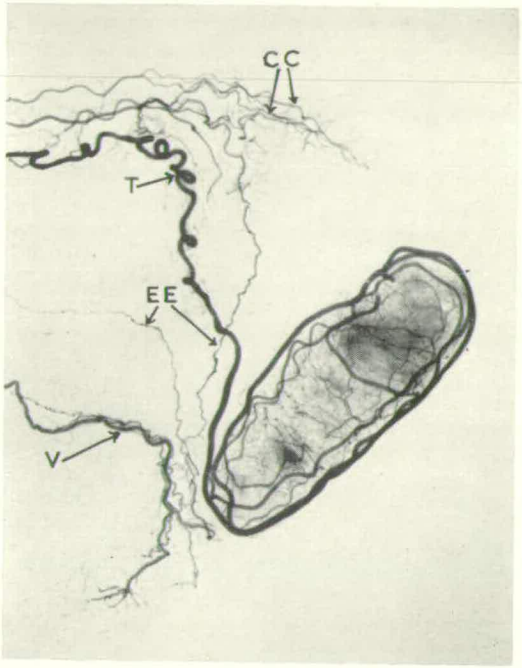


FIG. 1. Radiograph of the testis and epididymis of a rabbit after injection of the arteries with Micropaque. C.C. = capital arteries. T. = testicular artery. E.E. = descending epididymal arteries. V. = vasal artery.

(*Facing p. 123*)

corpus epididymidis lies in close proximity to the testis throughout most of its length, but in this species it ultimately continues posteriorly free from the testis. In this part, the convolutions of the tubule are intimately bound in spiral fashion by a fairly strong fibrous cord which extends from the inferior pole of the testis. The tubule next widens to become what is apparently the first part of the tail of epididymis, and a further widening gives rise to a large convoluted mass which turns forward and is attached to the more proximal convolutions by strong fascia. Finally the tubule continues as an independent ductus deferens (Text-fig. 1a). In the present experiment, interruption of the epididymis was effected opposite the inferior pole of the testis so that a short length of the body of the epididymis was isolated together with the tail of the epididymis and the vas deferens (see Text-fig. 1b). Therefore spermatozoa withdrawn from Point 4 in the diagram must be regarded as having been taken from the lower portion of the corpus epididymidis.

The blood supply to the upper parts of the epididymis in rabbits stems from the main testicular artery (see Pl. 1, Fig. 1). Several capital arteries pass to the head of the epididymis, whereas the body of the tubule is supplied with blood via separate descending vessels. A relatively discrete artery runs roughly parallel to the antero-lateral surface of the body of the epididymis, and a smaller vessel courses down the ventro-medial border. These two arteries join the much larger vasal or deferential artery on the surface of the cauda epididymidis. Despite interruption of the descending epididymal arteries, therefore, the distal part of the body of the epididymis and the tail should receive an adequate blood supply (see also Pl. 1, Fig. 1).

The course of the testicular artery in the rabbit has previously been described in detail (Harrison, 1948) and its superficial position on the surface of the testis is worthy of note in carrying out ligations of the epididymis, because the vessel runs close to the epididymis on the dorso-lateral surface of the testis. Caution was therefore to be observed in order to avoid damage to this artery when attempting a low ligation of the epididymis.

METHODS

Fourteen rabbits were used in the experiment; they were of mixed strain, varied from 2.0 kg to 3.2 kg in weight, and were housed in wire mesh cages at 68° F. Constant access to drinking water and food (Diet 18, as supplied by Oxo Ltd) was provided. Rabbits were anaesthetized by intravenous injection of sodium pentobarbitone (Veterinary Nembutal solution, Abbott Laboratories) by the method described by Cross (1955).

To isolate the distal part of the epididymis, two ligatures were applied around the body of the epididymis immediately above the tail, and the intervening segment removed. This was carried out unilaterally in the abdomen of each rabbit and the testis and epididymis secured in an abdominal position as previously described (Glover, 1958). The contralateral epididymis was ligated in the scrotum, so that the structures remained *in situ*. By this means each animal acted as its own control. Ten of the rabbits were sacrificed at 2-day intervals between 3 and 21 days after operation. As a check on the response, the

remaining four rabbits were killed at random intervals between these days, namely on the 4th, 8th, 10th and 14th days after operation. Spermatozoa were withdrawn from four different levels of the tubule in each rabbit as illustrated in Text-fig. 1b.

For examination of the spermatozoa, the tubule was carefully cut at the required level and the incised portion lightly touched with a microscope slide. Aqueous nigrosin-eosin solution (eosin Y [G. T. Gurr] 1.67 %, nigrosin [G. T. Gurr] 10.0%, dissolved in distilled water, 30 ml being made up fresh as required) was added to the spermatozoa on the slide before a smear was made. When the tail of the epididymis was incised, its contents flowed out freely and six drops of stain were added. But spermatozoa did not emerge so profusely from the vas deferens or more proximally from the narrow part of the tubule close to the ligature; for these samples, therefore, one or two drops of stain were adequate for obtaining a satisfactory distribution of spermatozoa on the slide for counting. On each slide, the percentage of dead spermatozoa was estimated together with the incidence of decapitated spermatozoa and the proportion of spermatozoa with coiled tails. Two separate counts of 100 spermatozoa were made on each slide wherever possible.

RESULTS

In order to assess the overall response of spermatozoa in the cauda epididymidis to artificial cryptorchidism, estimated mean values for the incidence of dead and decapitated spermatozoa taken from two levels within the region (Levels 2 and 3) were used. Estimates of the percentage of spermatozoa with coiled tails were treated similarly. For comparing the response at four different levels of the tubule (see Text-fig. 1a), mean values in groups of animals were taken, as illustrated in Tables 1 and 2.

THE EFFECT OF ARTIFICIAL CRYPTORCHIDISM ON SPERMATOZOA IN THE ISOLATED CAUDA EPIDIDYMIDIS

Mean values for characteristics of spermatozoa from two levels of the tail of the epididymis showed that an increased proportion of dead or eosinophilic spermatozoa occurred in the region after about 8 or 9 days of experimental cryptorchidism. Moreover, the percentage of dead cells increased progressively with the time of retention in the abdomen, though quantitative variation of response in individuals was considerable. Similar results were obtained from estimates of the incidence of decapitated spermatozoa in the cauda epididymidis. Quantitatively, the effect of decapitation was not as conspicuous as that of eosinophilia, but despite this, the trend of a progressive increase in the occurrence of decapitated forms was undoubtedly a real one.

Mean values for spermatozoa with coiled tails indicated that the incidence of this abnormality in the cauda epididymidis was not significantly influenced by the conditions of artificial cryptorchidism when the tubule was ligated.

In samples taken from control organs up to 21 days after operation, no change

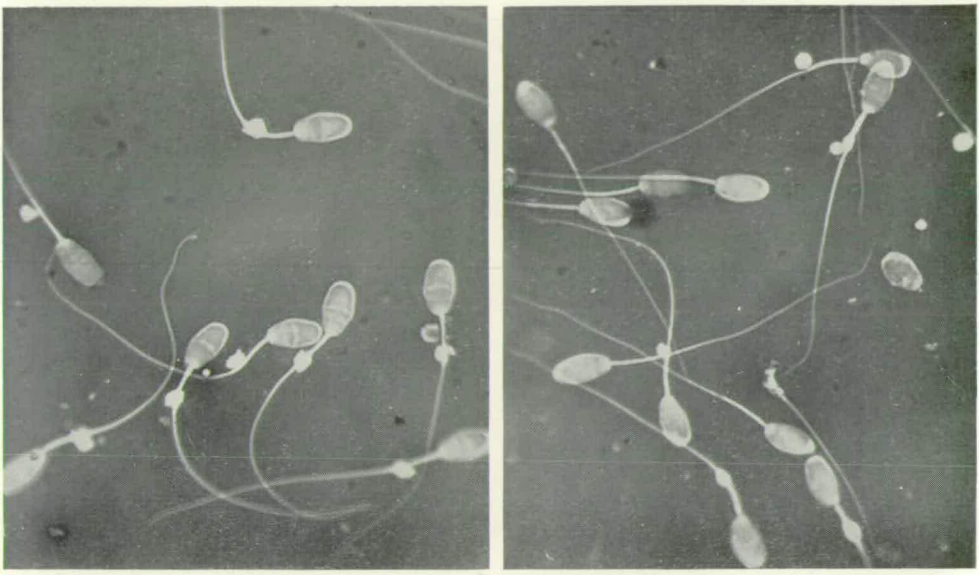


Fig. 2

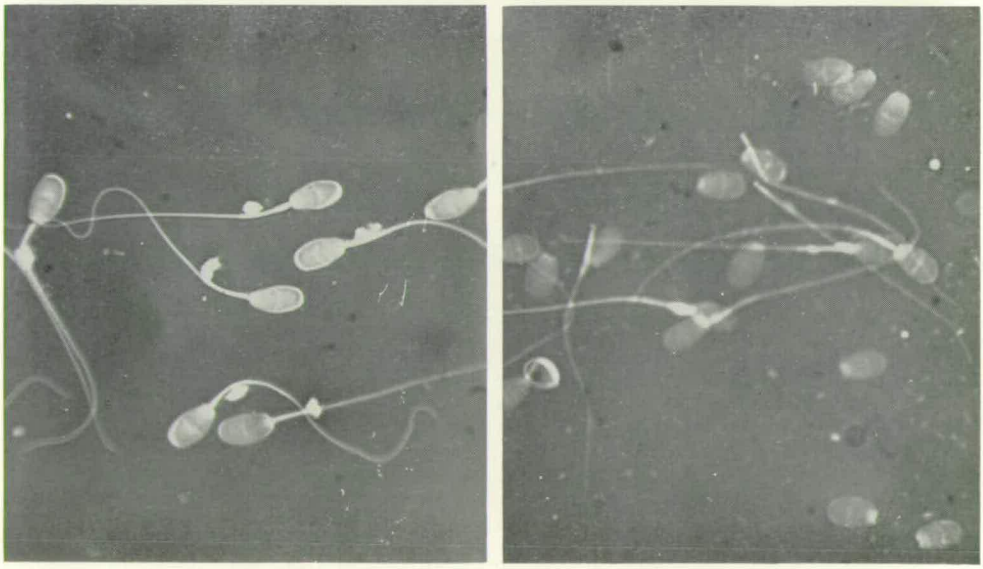
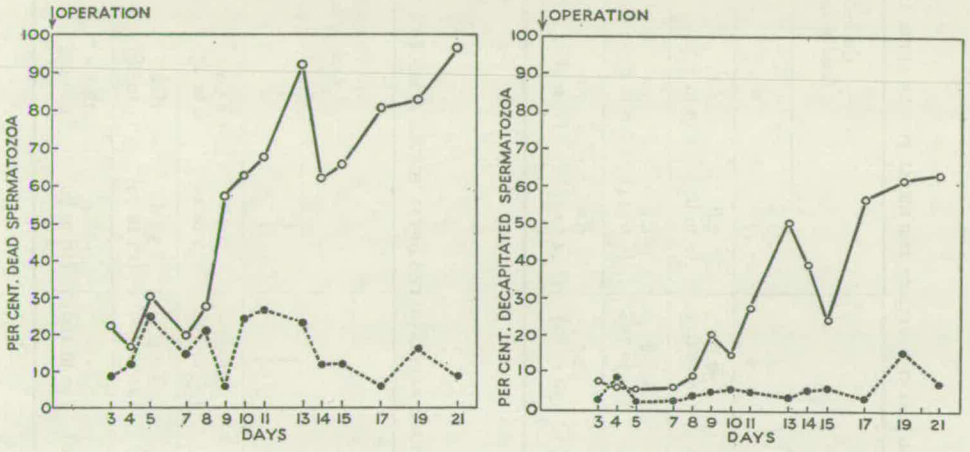


Fig. 3

Spermatozoa taken from the cauda epididymidis. *Left*: control side. *Right*: cryptorchid side. Cells stained with nigrosin-eosin. ×990.
FIG. 2. Seven days after isolation.
FIG. 3. Fourteen days after isolation.

(*Facing p. 125*)

in the morphology of spermatozoa or in the incidence of potentially active cells could be demonstrated. Hence, in animals destroyed up to about 7 days after operation, no very clear difference was evident between spermatozoa from abdominally retained epididymides and those from the control sides. After this time, however, the effects of abdominal retention became increasingly obvious (Pl. 2, Figs. 2 and 3). These results are illustrated graphically in Text-figs. 2a and 2b.



TEXT-FIGS. 2A AND 2B. *Characteristics of spermatozoa from the tail of the epididymis.* Mean values have been used of estimates from Levels 2 and 3 (see Text-fig. 1). Cryptorchids = ———. Controls = - - - - - - - -.

If it were assumed that in spite of ligation of the epididymis, migration and evanescence of spermatozoa in the distal part of the tubule continued, it might be argued that degenerate spermatozoa originated at a point proximal to the cauda epididymidis (viz. at Level 4) and were transported to lower levels. For this reason, quantitative estimates were made of the characteristics of spermatozoa from four different levels of the tubule at various stages after operation. These results are shown in Tables 1 and 2.

THE RESPONSE OF SPERMATOZOA AT DIFFERENT LEVELS OF THE TUBULE DISTAL TO THE AREA OF LIGATION

Mean percentages of dead and decapitated spermatozoa shown in Tables 1 and 2 indicate a rather higher degree of sperm degeneration at Level 3 (proximal part of the cauda epididymidis) than at Level 2 (distal region of the cauda epididymidis) in animals killed up to 8 days after their operation. The differences in estimates are, however, not statistically significant at the 5 % level using the test described by Moore (1957). On the same basis, the proportion of decapitated spermatozoa recovered from vasa deferentia did not exceed that in samples withdrawn from epididymides, except during the period of 9 to 14 days after ligation in control organs. Nevertheless, more dead spermatozoa were evident in the vas deferens of controls than in the cauda epididymidis between 9 and 21 days after ligation.

TABLE 1

THE INCIDENCE OF DECAPITATED SPERMATOZOA WITHDRAWN FROM DIFFERENT LEVELS OF THE DISTAL TUBULE AFTER LIGATION

		Cryptorchids *Level of tubule*				*Controls* *Level of tubule*			
		1	2	3	4	1	2	3	4
Estimates from 5 animals, 3 to 8 days after ligation	Mean (%)	4.4	6.0	9.4	32.0	3.8	1.2	4.8	32.2
	Range	(2 to 8)	(4 to 10)	(5 to 11)	(16 to 84)	(0 to 8)	(0 to 4)	(1 to 14)	(14 to 54)
Estimates from 5 animals, 9 to 14 days after ligation	Mean (%)	33.4	31.0	29.8	54.2	9.6	3.4	4.0	45.4
	Range	(16 to 44)	(16 to 52)	(11 to 48)	(12 to 76)	(6 to 14)	(0 to 6)	(0 to 9)	(18 to 74)
Estimates from 4 animals, 15 to 21 days after ligation	Mean (%)	55.7	48.0	54.0	53.7	9.2	7.0	6.5	42.5
	Range	(42 to 65)	(28 to 58)	(21 to 80)	(36 to 82)	(2 to 18)	(2 to 16)	(3 to 15)	(24 to 60)

TABLE 2

THE PROPORTION OF DEAD SPERMATOZOA OBTAINED FROM DIFFERENT LEVELS OF THE DISTAL TUBULE AFTER LIGATION

		Cryptorchids *Level of tubule*				*Controls* *Level of tubule*			
		1	2	3	4	1	2	3	4
Estimates from 5 animals, 3 to 8 days after ligation	Mean (%)	24.4	17.6	25.6	56.0	19.6	15.0	20.0	64.6
	Range	(12 to 38)	(10 to 26)	(20 to 45)	(36 to 100)	(9 to 36)	(6 to 23)	(12 to 28)	(43 to 100)
Estimates from 5 animals, 9 to 14 days after ligation	Mean (%)	74.0	70.8	69.4	86.4	33.8	16.8	22.4	78.0
	Range	(68 to 86)	(58 to 98)	(52 to 95)	(44 to 100)	(22 to 70)	(4 to 28)	(6 to 32)	(40 to 98)
Estimates from 4 animals, 15 to 21 days after ligation	Mean (%)	94.2	81.0	84.0	82.0	38.2	12.2	15.5	69.7
	Range	(79 to 100)	(65 to 94)	(70 to 100)	(40 to 100)	(16 to 80)	(4 to 23)	(7 to 31)	(36 to 100)

In the cryptorchid condition, the percentage of dead and decapitated spermatozoa in samples taken from Level 4 (distal part of the corpus epididymidis) was generally greater than in those withdrawn from Level 3 up to a period of 14 days. This was not observed in animals killed between 14 and 21 days. In control organs, however, an excess of degenerating spermatozoa in the body of the epididymis just distal to the ligatures was characteristic and the differences in estimates at Level 4 and Level 3 were thus clearly significant throughout.

Quantitative estimates of the characteristics of spermatozoa above the ligatures are as yet incomplete, but it may be stated here that considerable death and decapitation was noted in most abdominally retained epididymides.

DISCUSSION

Confirmatory evidence has been produced in this experiment that degenerate spermatozoa are to be found in the isolated cauda epididymidis of rabbits as a result of its retention in the abdomen; it is also clear that the onset of degeneration of the spermatozoa is delayed. That many spermatozoa were obtained from different levels of the isolated area of the tubule suggests that adverse effects of artificial cryptorchidism act upon spermatozoa throughout the entire length of the distal part of the tubule, and that the appearance of degeneration is not wholly due to the interchange of spermatozoa from one level to another.

The index primarily used for the response of spermatozoa was an assessed proportion of dead and decapitated spermatozoa; admittedly, metabolic changes which might not be recognized on this basis could precede the demonstrated response and thereby influence fertility at an earlier stage. Nevertheless, more actively motile spermatozoa could be recovered from these lower regions of the epididymis during the first week of retention in the abdomen than subsequently, and it would thus seem that spermatozoa in the cauda epididymidis possess a certain resistance to the effects of artificial cryptorchidism. Even when spermatozoa began to succumb to the adverse conditions, degeneration appeared to be a gradual and progressive process.

Estimates of the characteristics of spermatozoa taken from two levels of the cauda epididymidis indicated that the response to cryptorchidism was similar throughout the tail of the epididymis. It would seem, therefore, that in experimental cryptorchidism the characteristics of spermatozoa taken from any one point in the tail of the epididymis might be fairly representative of those throughout the whole area. At the same time, it is to be remembered that in intact animals some interchange of spermatozoa from the body of the epididymis might occur which could effect quantitative estimates in samples withdrawn from the cauda epididymidis.

A higher incidence of dead spermatozoa in the vas deferens than in the cauda epididymidis of controls after 9 days of ligation is to some extent commensurate with the suggestion of Young (1931) that spermatozoa in the vas deferens are the most aged. But conclusions drawn from the present experiment need to be tempered by consideration of the possible influence of ligation of the tubule.

A distinct preponderance of both dead and decapitated spermatozoa was apparent in most samples taken from the lower part of the body of the epididymis up to 14 days of experimental cryptorchidism. That this was not evident after longer periods of abdominal retention was probably due to an overall increase in dead and decapitated spermatozoa throughout the tubule which could overshadow any original excess in this area. At first glance, the result might seem to indicate a greater susceptibility of more immature spermatozoa to the effects of cryptorchidism, particularly when it is recalled that sperms in the body of the epididymis proximal to the ligatures showed a high degree of degeneration after retention in the abdomen. Yet while this may be so, the imputation is confused by considerable disintegration occurring in the corpus epididymidis of controls just distal to the ligatures.

This finding lends itself to a number of interpretations. It is tempting to dismiss disintegration near the ligatures as being purely due to trauma, but more interesting possibilities cannot on the present evidence be altogether excluded.

Obstruction of the normal flow of spermatozoa by the method adopted in this experiment might in itself cause spermatozoa immediately below the ligatures to degenerate. Though perhaps a little imaginative, the suggestion might be made that by virtue of an interrupted sperm stream a normal process of wastage of spermatozoa in the lower part of the corpus epididymidis could be revealed, which under normal circumstances might be hidden from view by an overwhelming number of normal spermatozoa perpetually descending the ductus epididymis. These possibilities are now being further explored.

The present work does not indicate whether the degeneration of spermatozoa was directly due to an increased environmental temperature, but this could be tested by using an alternative method of raising the temperature of the epididymis. Whatever the cause, however, the observed degeneration of spermatozoa in the tail of the epididymis is not in accordance with a number of previous claims. Phillips & McKenzie (1934) believed that, in the guinea-pig, artificial cryptorchidism exerted no effect upon the morphology of spermatozoa in the epididymis; this would suggest that morphological abnormalities of spermatozoa resulting from experimental cryptorchidism originate entirely in the testes. Gunn (1936) found that, in rams, decapitated spermatozoa resulting from insulation of the scrotum appeared in ejaculates at a time which corresponded to the passage of indian ink through the epididymis. He therefore concluded that the delay in appearance of these abnormalities represented the time of migration of spermatozoa through the epididymis. Walton & Uruski (1946) reached similar conclusions after subjecting rabbits to low atmospheric pressures. The present results do not substantiate such a claim being made as far as experimental cryptorchidism in rabbits is concerned. As has been previously suggested (Glover, 1959), some of the first decapitated spermatozoa found in the ejaculates of cryptorchid rabbits are likely to originate in the tail of the epididymis. To this it may now be added that the vas deferens provides another place of origin of disintegrating spermatozoa. It is therefore suggested that whilst the delay in appearance of degenerating spermatozoa in ejaculates of rabbits after artificial induction of cryptorchidism might prove to coincide with

the time of migration of spermatozoa through the tubules of the epididymis, it is rather an indication of the resistance of so-called mature spermatozoa to the effects of retention in the abdomen.

ACKNOWLEDGMENTS

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Disintegrated Spermatozoa from the Epididymis

In the course of an investigation of the response of spermatozoa in the epididymis of rabbits to the effects of artificial crytorchidism, it was noted that in the controls (that is, 12 normal epididymides on which no operative interference had been undertaken) a remarkable number of immotile decapitated spermatozoa was present in samples withdrawn from regions of the tubule proximal to the cauda epididymidis. Spermatozoa of this kind have been previously referred to as 'disintegrated spermatozoa'[1,2] and presumably indicate a process of degeneration. In the present work the morphology of spermatozoa was examined in saline-diluted squash preparations under the phase-contrast microscope and in whole specimens. Quantitative estimates of particular characteristics were made from smears after staining with aqueous nigrosin eosin differential stain[3] in order that an estimate of the proportion of 'dead' (eosinophilic) spermatozoa also might be made. In view of this finding, spermatozoa from epididymides of some other species, namely the bull (6 specimens), the goat (2 specimens), the dog (4 specimens) and the cat (3 specimens) were also examined.

In all these cases a proportion of spermatozoa withdrawn from the vas deferens appeared to be degenerate; but it was possible to discern more evidence of disintegration in samples from the body of the epididymis. A variable percentage of dead and decapitated spermatozoa was observed in smears of samples taken from the head of the epididymis and from the initial segment[4]. These percentages were, however, generally lower than those in samples from the body of the epididymis. A relative paucity of degenerate forms from the tail of the epididymis in most specimens was typical. In bovine specimens the overall degree of disintegration of spermatozoa from all levels of the epididymis was comparatively small, but more eosinophilic spermatozoa were present in samples from the body of the epididymis than from any other level, which confirms an earlier report by Ortavant[5]. In both ruminants, differences between the incidence of disintegrated spermatozoa from the body of the epididymis and the cauda epididymidis were not conspicuous. Differences in mean values obtained for the incidence of decapitated spermatozoa from these two areas were, for the bull 8 per cent, and for the goat 16 per cent. In the dog, cat and rabbit, however, the contrast between samples from these two areas was striking, there being many more disintegrated spermatozoa from the body of the epididymis. Differences in mean values obtained

between samples from the two parts in each of these species respectively were, 23, 66 and 49 per cent for decapitated spermatozoa, and 36, 63 and 61 per cent for eosinophilic spermatozoa. Despite considerable ranges of variation, differences between estimates from the two levels in these species were plainly significant.

From the observations it is not clear what proportion of disintegrated spermatozoa was due to artefact. The occurrence of disintegrated forms in whole samples where neither staining nor smearing was involved indicates that a number of spermatozoa were degenerate within the intact tubule of the epididymis, and thus some degree of wastage of spermatozoa in proximal regions of the epididymis is apparent. But in stained smears, it is conceivable that separation of the heads in several spermatozoa might have occurred *in vitro* and might thereby represent partly a variation in fragility of the cells at different levels of the tubule. In this connexion, it has been reported that decapitation of spermatozoa in ejaculated semen can be produced by certain methods adopted for the staining and clearing of smears[6]. Alternatively, differences in the viscosity of samples could interfere with the free movement of spermatozoa across the slide and give rise to an increased incidence of decapitated spermatozoa in smeared preparations. Subjectively, the milieu of spermatozoa from the tail of the epididymis appears to differ from that in the more proximal regions, especially in rabbits, and this might be related to possible differences in the effects of handling. Such variation in medium might also allow disintegrated spermatozoa to be obscured in smears of samples taken from the cauda epididymidis due to the effects of a specific distribution of degenerate forms. However, that the highest proportion of disintegrated spermatozoa was found throughout in samples from the body of the epididymis is of interest in that it suggests at least some characteristic quality of the tubular contents at this level. Experiments are now in progress to determine more precisely the inference to be drawn from this surprisingly regular pattern in the incidence of disintegrated spermatozoa from different areas of the epididymis.

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THE REACTION OF RABBIT SPERMATOZOA TO NIGROSIN EOSIN FOLLOWING LIGATION OF THE EPIDIDYMIS

T. D. Glover, Ph.D. (England)

IT is generally accepted that mammalian spermatozoa undergo process of maturation as they migrate through the epididymis. How-ver, Ortavant[1] showed that in bulls, if epididymal spermatozoa were tained with a mixture of eosin and aniline blue, those taken from ne portion of the body of the epididymis absorbed eosin more readily han did spermatozoa from any other area of the tubule. Similar ndings in a variety of mammalian species have since been reported sing aqueous nigrosin as the stain* (Glover[6]). In this recent work, xtra fragility of spermatozoa from the body of the epididymis was lso indicated since the incidence of disintegrated forms from this rea was considerable in some species, notably in the rabbit, dog, and at.

It seems reasonable to believe, therefore, that, as Ortavant ostulated, a critical phase in the maturation of spermatozoa might xist. The evidence suggests, at least, that the concept of maturation eing a steadily progressive process might require some modification, nd it was decided that the whole problem demanded further ex-loration. The experiment to be described was designed for this urpose, in the belief that the reaction of spermatozoa to interference ith their normal migration might be of value in the interpretation f events occurring in intact epididymides. Some information as to he effects of ligation of the epididymis in rabbits had already been btained from a different experiment (Glover[7]), and the present work as undertaken to provide additional data. Nigrosin eosin was again sed for staining samples of spermatozoa, and by means of unilateral gation of the epididymis in each rabbit, opportunity could be taken

*This stain has been tested by several workers for the differentiation of live and ead spermatozoa in ejaculated semen of mammals (Blom,[2] Hancock,[3] Campbell, Hancock, nd Rothschild,[4] Campbell, Dott, and Glover[5]).

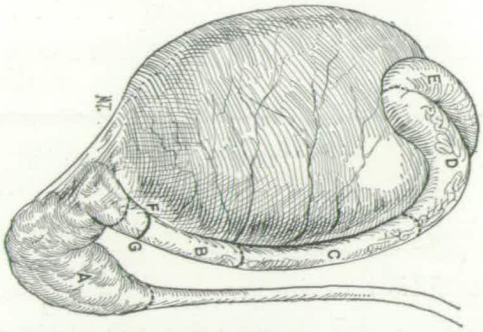
to compare, in the same animal, the reaction of spermatozoa from an intact epididymis with that of spermatozoa from a ligated tubule.

Materials and Methods

Ten rabbits of mixed strain were used in the investigation. They were housed, fed, and watered under constant conditions and they weighed from 2.8 to 3.4 kg. With the exception of one rabbit, which was left intact, ligation of the epididymis was carried out unilaterally in each animal, contralateral organs being maintained intact to serve as controls. Ligation of the tubule was performed close to the distal extremity of the body of the epididymis. Young[8] in studying a similar problem in guinea pigs ligated the epididymis at a higher level, but in the present work spermatozoa from the body of the epididymis were of particular interest. Had ligation been carried out nearer to the testes, the corpus epididymidis would shortly have been emptied of spermatozoa owing to their migration, for, as Macmillan[9] showed in the rat, migration can proceed even in the absence of a *vis a tergo* from the testis.

For ligation of the epididymis, animals were anesthetized with sodium pentobartitone (Veterinary Nembutal solution, Abbott Laboratories). The operation was performed in each case through a small incision in the lateral aspect of the scrotum. The tunica vaginalis was incised and a single ligature taken round the epididymis. Care was observed to pass the suture beneath the descending vessels (see Glover[10]) so that the blood supply should not be impaired. In this way the epididymis was disturbed as little as possible, and all that was needed to complete the operation was a single suture, passed first through the tunica vaginalis and then through the skin.

Three animals were sacrificed during the first week after the operation (on days 3, 5, and 7), three during the second week (on days 10, 12, and 14) and three during the third week (on days 17, 19, and 21). The tenth animal, upon which no operation was performed, was killed also on day 21 after the beginning of the experiment. After each rabbit was killed the testes and epididymides were removed and put in normal saline. Within five minutes they were removed from the saline, and spermatozoa were taken from different levels of the tubule for examination (see Figure 1). This was accomplished by incising the appropriate region of the tubule and smearing the emergent spermatozoa onto two microscope slides after mixing them with one or two drops of aqueous nigrosin eosin (nigrosin [G. T. Gurr] 10.0 per cent and eosin Y [G. T. Gurr] 1.67 per cent, dissolved in 30.0 ml. of distilled water). A total of 100 spermatozoa were counted on each side and the mean percentage of stained spermatozoa

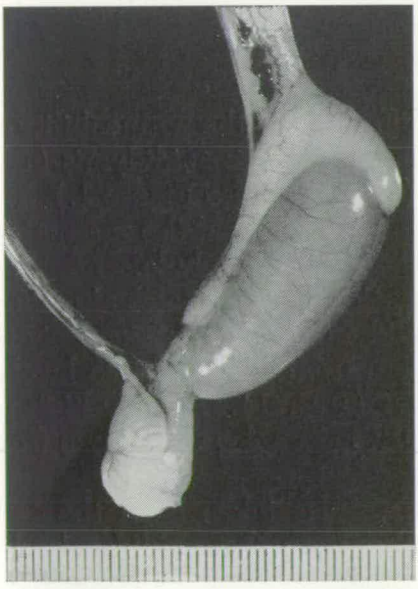


igure 1. Diagram of testis and epididymis of the rabbit showing the site of ligation (F-G) and the different areas from which spermatozoa were withdrawn.
A Cauda epididymidis (tail of epididymis)
B Lower part of corpus epididymidis (body of epididymis)
C Upper part of corpus epididymidis
D Caput epididymidis (head of epididymis)
E Initial segment

including decapitated forms) was used to represent the degree of osinophilia. On each occasion a fresh sample of spermatozoa was irst examined under the microscope for assessment of its motility.

Results

In control epididymides spermatozoa did not flow so freely rom the incised tubule in upper regions of the epididymis as they lid from the cauda epididymidis. This was particularly apparent at he initial segment and the body of the epididymis, from which re-ions, it was also observed, the samples appeared to be more viscous.



igure 2. Appearance of testis and epididymis of the rabbit two weeks after epididymal ligation. Note distention of the tubule above the ligature (see text).

Following ligation, however, these differences in the quality of sam ples from different levels of the tubule disappeared and many sper matozoa were easily obtained from all levels, particularly during th second week. This was accompanied by distention of the tubul above the point of ligation so that the convolutions of the epididymi became clearly distinct (see Figure 2). There was some variatio in the degree of distention at different levels; in some animals th convolutions were most prominent at the head flexure, while in other they appeared most distinct in the head of the epididymis proper However, it was evident that despite ligation of the epididymis, sperm production continued, at least for some time.

Spermatozoa from Control Epididymides

Estimates of the incidence of stained spermatozoa from dif ferent levels of control epididymides are given in Table I. It illus

TABLE I

Percentage of Eosinophilic Spermatozoa from Intact Epididymides

Region of the Epididymis					*Testis*
Tail (A)	*Lower Body (B)*	*Upper Body (C)*	*Head (D)*	*Initial Segment (E)*	
21	100	56	62	58	58
15	75	60	44	46	45
12	51	78	55	94	58
16	72	92	54	98	60
14	56	70	31	56	62
19	84	92	25	72	57
13	100	94	66	100	55
15	70	65	41	82	68
19	82	66	44	85	55
14	75	52	40	64	60
18	77	70	46	62	66

trates the dramatic difference in the permeability to this stain betwee spermatozoa from the body of the epididymis (B) and those from the cauda epididymidis (A). (See also Figure 1.) This change in perme ability appears to be quite sudden as the spermatozoa pass the junctio between the two regions of the tubule.

With one exception, the estimates also show that samples from the head of the epididymis (D) contained significantly less stained spermatozoa than those from either of two levels of the body of the epididymis (B and C) ($P < .01$). A fairly high proportion of stained cells occurred, however, in samples withdrawn from the initial segment (E).

Estimates on samples taken from the testes are less reliable than those on samples from the epididymis, because considerable cellular debris was apparent on slides made from testicular samples and differentiation of stained spermatozoa from unstained ones was

difficult, there being many examples of partial or incomplete staining. However, it does seem that a considerable proportion of spermatozoa from the testes had affinity for the eosin.

In the assessment of motility the simple microscopic observations that were made did not prove ideal for discerning small differences between samples from different levels of the tubule. Nevertheless, over-all motility was undoubtedly greatest in samples taken from the tail of the epididymis. But it should be pointed out that samples from this region contained the highest total number of spermatozoa, and in addition, many active spermatozoa were to be seen in samples from higher levels of the tubule. Most of these spermatozoa had proximally situated protoplasmic droplets and could thus be regarded as immature.

Spermatozoa from Ligated Epididymides

During the first week of ligation the mean incidence of eosinophilic spermatozoa from level B (that is, immediately proximal to the ligature) was 42 per cent (see Table II), but it rose in samples

TABLE II

The Incidence of Eosinophilic Spermatozoa From Ligated Epididymides

Weeks After Ligation	*No. of Animals*		*Region of the Epididymis*					
			Tail (A)	*Lower body (B)*	*Upper body (C)*	*Head (D)*	*Initial segment (E)*	*Testis*
1	3	Mean %	15	42	32	23	53	60
		Range	(12-18)	(29-56)	(24-43)	(18-32)	(50-56)	(46-74)
2	3	Mean %	12	67	32	53	44	35
		Range	(8-17)	(66-86)	(22-51)	(42-62)	(35-54)	(29-42)
3	3	Mean %	17	53	32	35	19	20
		Range	(14-22)	(45-62)	(28-37)	(20-50)	(14-23)	(16-23)

taken during the second week, and a fairly high level was maintained during the third week. By contrast, the proportion of stained spermatozoa from level C remained relatively constant. In samples taken from the head of the epididymis and the initial segment (D and E) the incidence of stained cells was distinctly low on the twenty-first day. In samples from the initial segment, sequential estimates showed also a gradual decline in the proportion of stained spermatozoa. These effects are illustrated graphically in Figure 3, where the mean of estimates from levels D and E emphasizes the decrease in eosinophilia which occurred progressively in sperm populations withdrawn from upper regions of the tubule proximal to the corpus epididymidis.

Motility in all these samples was markedly greater than in samples from comparable regions of intact epididymides, and the number of actively motile spermatozoa taken from the testes was un-

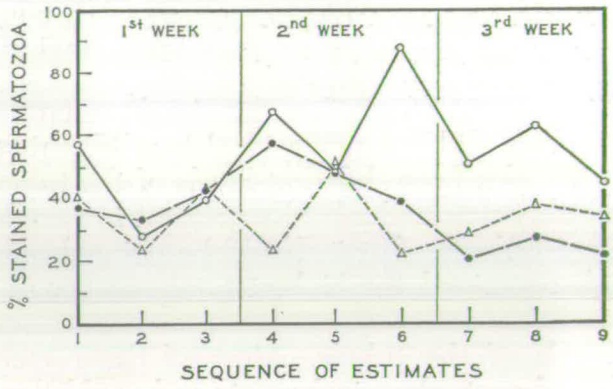


Figure 3. Incidence of stained spermatozoa following ligation of epididymis.
o———o Lower part of body (B)
Δ— — —Δ Upper part of body (C)
• — — — • Mean value from head of epididymis and initial segment (D and E).

questionably increased. From the second week onward, seminiferous tubules near the proximal pole of the testis could be seen macroscopically to be prominently distended. Incision of this area of the testis revealed a profusion of spermatozoa not usually present in such quantities. These were mostly non-eosinophilic.

Discussion

The results of this experiment provide further evidence that spermatozoa of the rabbit undergo a characteristic change in their surface nature as they pass into the body of the epididymis. This change, however, is overcome by the time the cells reach the tail of the epididymis, where they are regarded as mature. It would seem, therefore, that the change in permeability which permits the absorption of eosin is reversible in vivo and represents merely a stage in the process of maturation of the spermatozoa. Otherwise, the typical rejection of eosin by spermatozoa from the tail of the epididymis following nigrosin eosin staining would be difficult to explain.

If the change is only a temporary event, the question arises as to what effect low ligation of the tubule might have upon spermatozoa in the body of the epididymis, since theoretically they should be precluded from undergoing the final stage of maturation. Young[8] showed, however, that high ligation of the epididymis in guinea pigs resulted in increased motility and fertilizing capacity of spermatozoa removed from areas of the tubule above the site of ligation. These effects gradually increased up to twenty-five days and led to the conclusion that spermatozoa could mature without migration. In addition, it was suggested that maturation was an intrinsic property of the cells

nd that time might be one of the most important factors in its occurrence. These ideas were not in accordance with the beliefs of arlier workers, including Tournade[11] and Redenz,[12] who considered naturation to be primarily due to the action of epididymal secretions. Iore recently, Salisbury[13] expressed the view that both factors are kely to be involved. The evidence suggests, however, that, whatever ne cause, maturation changes might be expected following ligation f the epididymis. Thus the eosinophilic nature of spermatozoa in the ody of the epididymis might be expected to disappear following gation.

Present results have shown that this does not altogether take lace. From ligated tubules, prominently distended with fluid and permatozoa, the cells that were removed exhibited very active notility on exposure to the atmosphere, and the majority of those vithdrawn from the head of the epididymis, the initial segment and ne testes were refractory to eosin when stained with differential tain. In samples taken from the body of the epididymis, however, nany spermatozoa were seen to have retained their permeability to osin although the incidence of stained cells was less than in comarable samples from the controls.

The problem that next arises is the extent to which the rejecon of eosin by spermatozoa could be regarded as a sign of their naturity. In this experiment there was a somewhat arbitary relationhip between the motility of a sample and the proportion of unstained permatozoa in that sample. Also, it was observed, several spermatozoa from the initial segment and the testis exhibited very active notility in vitro despite the presence of a proximal cytoplasmic roplet. These findings are not completely in accordance with those f Branton[14] on bull spermatozoa, but it is to be recalled that in the resent work, motility estimations were carried out after dilution, nce a considerable quantity of saline remained on the epididymis efore it was incised. It seems, however, that the term "maturation" somewhat nebulous in the context of this experiment, so for the emainder of the discussion reference will be confined to the two oberved effects, namely changes in the motility of samples and in the ermeability of spermatozoa to eosin.

A possible interpretation of the reduction in the proportion of tained spermatozoa following ligation of the epididymis could be nat there was an intrinsic change in the permeability of the cells to ne stain. This might be considered to receive support from the radual development of eosinophilia in spermatozoa immediately bove the ligature, for this could represent aging of the spermatozoa rapped in the tubule. The body of the epididymis could thus be egarded as having taken over the role normally played by the tail

of the epididymis and the vas deferens. But an increased number of unstained spermatozoa occurred at all levels of the tubule and in the testis as early as three days after ligation. It seems unlikely, therefore, that a capacity of the spermatozoa to become motile in vitro and also to reject eosin was intrinsic, when it is remembered that in the normal animal they are first required to traverse practically the entire epididymis.

If extrinsic factors were also involved, changes in the surface nature of spermatozoa in a ligated epididymis might be explained as being due to the accumulation of excess epididymal fluid. This would seem quite likely, for the effect could be a rapid one. As regards spermatozoa from the testis, however, a backflow of epididymal secretion would have to be postulated unless an abundance of testicular fluid could itself induce a potential capacity in the spermatozoa for motion in vitro as well as a change in their permeability to eosin.

Finally, as far as spermatozoa in the body of the epididymis were concerned, the possibility that ligation of the tubule evoked no significant change in the nature of individual spermatozoa cannot be ignored. In control epididymides many of the spermatozoa from the head of the epididymis were unstained, and it is probable that after ligation some migration of spermatozoa continued, for a time at least. This would give rise to an increased number of spermatozoa at all levels of the epididymis, and in spite of an obvious increase in the amount of fluid following ligation, this appeared to occur. If, under these conditions, the number of stained spermatozoa remained the same, the number (and proportion) of unstained cells would be increased in direct relation to the total increase in the population.

The argument could also apply to increased motility of samples withdrawn from the body of the epididymis after ligation. However, to use this as an explanation for changes in spermatozoa from more proximal areas of the epididymis and particularly in those from the testis is more difficult. It would necessitate an assumption that many spermatozoa have already acquired the capacity for motion in vitro and for the rejection of eosin by the time they leave the testis. That some of them do this has been indicated by the present results, but even if the possibility were accepted, the great majority of stained spermatozoa from the testis and body of the epididymis of the controls would present a formidable problem in interpretation. It might be that spermatozoa migrate at very different rates and that stained spermatozoa are those which have failed to migrate satisfactorily. This would explain their virtual absence in the tail of the epididymis in normal animals. At this stage of our knowledge such a consideration cannot be altogether excluded.

It is evident that many complex problems associated with the function of the epididymis remain to be solved. With the advent of

modern chemical techniques much should be accomplished in the future, while some aspects of the subject might be explored by other methods. The present experiment, therefore, can be considered only as a prelude to more extensive investigations.

Summary

Some effects of experimental interruption of the migration of spermatozoa have been investigated by subjecting adult male rabbits to unilateral ligation of the epididymis. Animals were sacrificed at varying intervals during a three-week period after operation and spermatozoa were withdrawn from specific sites throughout the epididymis. The motility of spermatozoa was assessed microscopically and further examination of samples included the use of aqueous nigrosin eosin as a differential stain.

Results have shown that following ligation of the epididymis, spermatozoa from regions of the tubule above the point of occlusion appeared to exhibit increased motility in vitro. But nigrosin eosin smears revealed the presence, in these samples, of a number of spermatozoa that had absorbed eosin from the stain. The incidence of these types of spermatozoa decreased with the time of ligation in samples taken from the testis and upper regions of the epididymis, while their proportion increased in samples from the lower part of the body of the epididymis at a site immediately proximal to the ligature.

The results are discussed in relation to the findings in intact control epididymides.

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THE RESPONSE OF RABBIT SPERMATOZOA TO ARTIFICIAL CRYPTORCHIDISM AND LIGATION OF THE EPIDIDYMIS

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SUMMARY

By means of unilateral ligation of the epididymis and experimental cryptorchidism in twenty-one adult male rabbits, the reaction of spermatozoa in proximal regions of the epididymis to the effects of retention in the abdomen was investigated. Combined estimates of eosinophilic spermatozoa and decapitated spermatozoa in smears stained with aqueous nigrosin eosin were used to indicate the response in different samples.

Examination of spermatozoa withdrawn from four levels of the tubule showed that those in the caput epididymidis and the initial segment reacted rapidly to the effects of artificial cryptorchidism, whilst the response of spermatozoa from the corpus epididymidis appeared to be delayed.

Contralateral testes and ligated epididymides were maintained in the scrotum to serve as controls, and under these circumstances spermatogenesis continued for at least 3 weeks although, shortly after ligation, the epididymides were tightly packed with spermatozoa and fluid. Examination of the tubular contents in these specimens by means of the nigrosin eosin technique revealed a progressive degeneration of spermatozoa above the site of ligation. However, microscopical observations of motility indicated enhanced activity in samples collected during the first 3 weeks of ligation of the epididymis.

INTRODUCTION

From previous publications (Glover, 1955, 1959*a*) it is evident that insulation of the scrotum in rams and artificial cryptorchidism in rabbits result in almost identical changes in the morphology of ejaculated spermatozoa. One characteristic feature of these changes is the appearance of decapitated spermatozoa in ejaculates after about 1 week of continuous treatment, and the similarity in effect suggests that similar conditions might prevail in both cases. Both procedures are known to cause an increase in the temperature of the testes and this, whether directly or indirectly implicated, might be an important common factor. There is already evidence, however, that whatever causes decapitation of spermatozoa in artificial cryptorchidism it is not restricted in its effects to the testes but also involves spermatozoa in the epididymis (Glover, 1959*b*). Furthermore, it has been shown that spermatozoa in the isolated cauda epididymidis of rabbits tend to be decapitated only after 8–9

days of artificial cryptorchidism (Glover, 1960). In these conditions, therefore, the effect appeared to be cumulative. In rams, however, decapitation of spermatozoa can be induced, not only by continuous insulation of the scrotum but also by short (24 hr.) periods of treatment. A cumulative effect would not be possible under these circumstances, but it is to be noted that the decapitated forms did not appear in ejaculates for 17–24 days even in conditions of regular sexual activity.

Differences in procedure, and species variation, must be taken into account in interpreting these results, but if increased temperature is the vital common factor operating in all the cases, it could be that short periods of temperature elevation only affect spermatozoa in upper regions of the epididymis. The belated appearance of decapitated spermatozoa in the semen of rams following such short periods of treatment could then be accounted for by their migration through the epididymis. This would indicate that spermatozoa in proximal regions of the epididymis were more susceptible to the effects of elevated temperature than those in the tail of the epididymis. In favour of this possibility is the currently held view that spermatozoa in upper regions of the epididymis are relatively immature.

The purpose of the present experiment, therefore, was to determine the susceptibility of spermatozoa in proximal parts of the epididymis to the effects of increased temperature induced by means of artificial cryptorchidism. By ligation of the epididymis an attempt was made to arrest migration of the spermatozoa and thus avoid their movement from one level of the tubule to another. In each animal, whilst one testis and its associated epididymis was secured in the abdomen, the other was allowed to remain in the scrotum. In this way it was possible to assess also the effects of ligation of the tubule, in itself, upon the characteristics of contained spermatozoa, and thus facilitate a more accurate interpretation of data concerning samples from organs retained in the abdomen.

MATERIALS AND METHODS

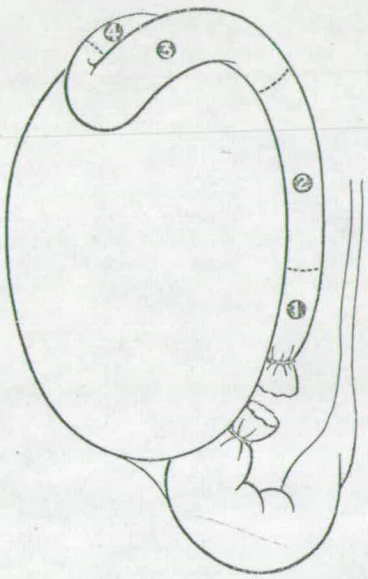
Twenty-one rabbits of mixed strain weighing from 3·2–3·5 kg. were used in the experiment. They were kept at a temperature of 68° F, had permanent access to drinking water, and were provided with the same diet (S.G. 1, Oxo, Ltd). All operations were performed under anaesthesia using slow intravenous injection of sodium pentobarbitone ('Nembutal' solution, Abbott Laboratories Ltd). Occlusion of the epididymis was carried out by means of ligation of the tubule near to the distal extremity of the corpus epididymidis. Details of this method have been given previously (Glover, 1960), but in the present work extra care was needed to preserve intact the blood supply to all parts of the tubule.

On one side of each animal the ligated epididymis and its associated testis was confined within the abdomen whilst the corresponding contralateral organs were retained in the scrotum to serve as controls. Rabbits were killed at varying intervals after operation and, for the analysis of results, were grouped according to the week in which they were killed (see Tables 1 and 2).

Spermatozoa were obtained from four levels of the epididymis above the point of occlusion as illustrated in Text-fig. 1, and corresponding areas of the tubule were removed also for histological examination. Immediately after death the testes and

epididymides were removed from each rabbit and placed in normal saline, and appropriate portions of the epididymis were cut out as required.

From each of these portions, one segment was separated and fixed in aqueous Bouin's solution while the open end of the remainder was held for a moment in each of two drops of aqueous nigrosin eosin differential stain (nigrosin (G. T. Gurr) 10·0 %, eosin Y (G. T. Gurr) 1·67 %, dissolved in distilled water) which had been deposited on separate microscope slides. By this means spermatozoa were mixed with stain on the slides and duplicate smears were made directly. The method was used because in preliminary tests it was noticed that if nigrosin eosin was added to



Text-fig. 1. Diagrammatic representation of the testis and ligated epididymis, showing areas which were removed for histological section and examination of spermatozoa. (1) Distal extremity of the body of the epididymis. (2) Upper region of the body of the epididymis. (3) Caput epididymidis. (4) Initial segment (designation introduced by Benoit (1926) for this region of the tubule).

samples already on the slides, the distribution of degenerated spermatozoa tended to be uneven when a smear was made and this was obviously undesirable for making quantitative estimates.

Degenerative changes in the spermatozoa were assessed by combining the estimated percentage of stained (eosinophilic) spermatozoa with the estimated incidence of decapitated spermatozoa. This was done largely in order that the two criteria could be compared under the conditions of this experiment, since each of them has been used separately in the past to indicate degradation of spermatozoa. A total of 100 spermatozoa was counted in random fashion on each slide, and the mean of both estimates was used in appraisal of the sample.

Occasionally additional smears were first made and stained with Harris's haematoxylin, residual saline on the tubule being sufficient to allow a satisfactory smear to be made without any further dilution. Samples were also examined from time to

Table 1. *Proportion of stained and decapitated spermatozoa from different levels of the epididymis after ligation and retention in scrotum*

Weeks after ligation of epididymis	No. of samples	Level of epididymis 1	2	3	4
		Mean percentage stained spermatozoa ±S.E.			
1	5	40·00±4·87	39·00±10·81	26·20±4·47	42·20± 6·80†
2	7	60·14±7·55*	40·43± 9·80	56·14±6·53**	47·06±10·21
3	5	55·00±4·73	31·20± 2·85	32·40±5·23−*	18·00± 5·65−*
5	4	81·00±6·95*	82·50± 7·42**	57·00±3·17**	33·56± 8·97*
		Mean percentage decapitated spermatozoa ±S.E.			
1	5	19·60± 1·21	17·20± 7·11	8·60± 3·79	16·40±4·92†
2	7	36·14± 6·23*	20·29± 8·03	21·14± 5·34*	12·57±2·11
3	5	39·80± 3·88	18·00± 2·53	20·00± 4·72	7·60±2·21
5	4	65·00±10·96*	71·00±12·19**	50·50±11·44*	18·00±3·40*

* Significant difference ($P < 0{\cdot}05$) between corresponding figure and that in preceding week at same level of epididymis. −* Where difference is one of reduction.
** Highly significant difference ($P < 0{\cdot}01$).
† Low overall incidence of spermatozoa.

Table 2. *Incidence of stained and decapitated spermatozoa from different levels of the epididymis after ligation and retention in abdomen*

Weeks after ligation of epididymis	No. of samples	Level of epididymis 1	2	3	4
		Mean percentage stained spermatozoa ±S.E.			
1	5	50·60+4·90	54·80±6·39	58·60±7·25	70·20±9·84†
2	7	82·28±6·66**	86·29±5·29*	91·00±4·55**	87·71±2·35
3	5	91·00±2·76	87·20±3·37	97·00±1·55	92·80±3·07
5	4	97·50±1·89†²	None	None	None
		Mean percentage decapitated spermatozoa ±S.E.			
1	5	30·80±6·53	38·80±9·30	37·00±10·25	51·40±10·64†
2	7	67·14±8·23*	68·71±8·61*	61·29± 6·25*	71·29± 4·02
3	5	80·00±7·28	68·00±7·56	78·50± 4·53	81·26± 5·04
5	4	90·00±0·96†²	None	None	None

* Significant difference ($P < 0{\cdot}05$) between corresponding figure and that in preceding week at same level of epididymis.
** Highly significant difference ($P < 0{\cdot}01$).
† Low overall incidence of spermatozoa.
†² Spermatozoa present in only two samples.

time under phase contrast without being either smeared or stained. These preparations were only used, however, as a rough check on the response of spermatozoa and quantitative estimates were not undertaken on these slides.

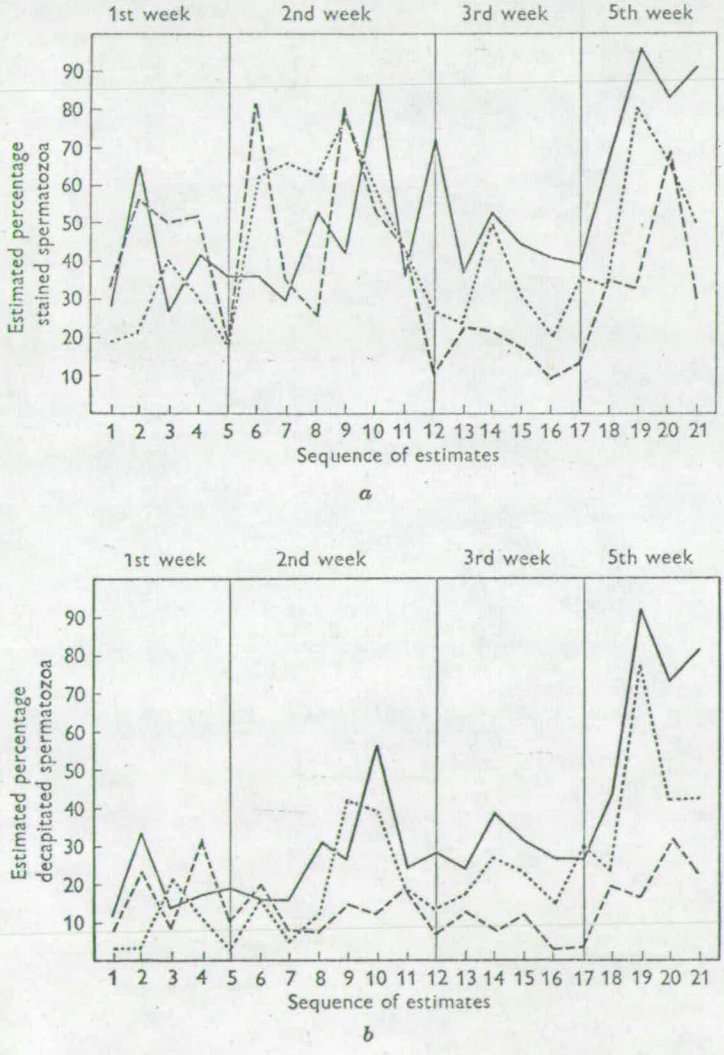
For histological examination of testes and epididymides, sections were cut at 7 μ and stained with Harris's haematoxylin, 1·0 % chromatrope 2R being used as a counterstain (see Lendrum, 1935).

RESULTS

From the data obtained it was clear that the interpretation of results from abdominally retained organs must be considerably influenced by events in control organs. For this reason, data from the controls is presented first.

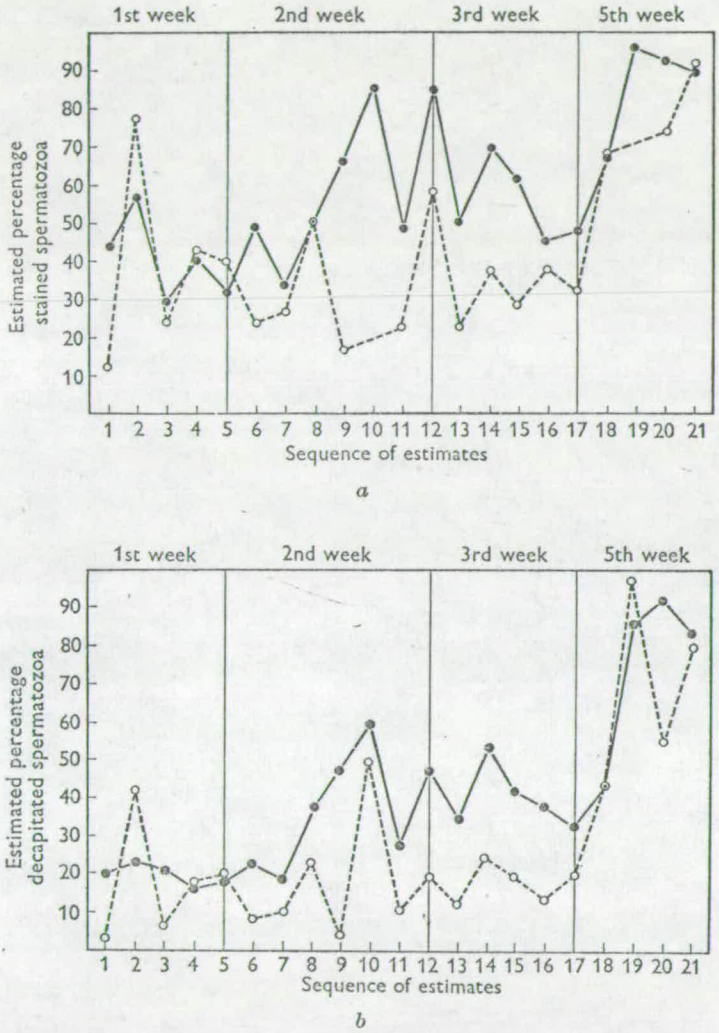
Influence of experimental occlusion of the ductus epididymidis

Control epididymides showed considerable distension above the ligature within 1 week of the operation. The convolutions of the tubule which, in upper regions of the rabbit's epididymis are normally indistinct when viewed with the naked eye, were



Text-fig. 2*a*, *b*. Incidence of stained spermatozoa and decapitated spermatozoa after ligation of the epididymis and retention in the scrotum. ——, Body of the epididymis (means of estimates from levels 1 and 2); – – –, head of the epididymis (level 3); . . ., initial segment (level 4).

prominent and of a vivid cream colour. These features were not always regular, however, and some tubules looked patchy where particular portions were excessively distended. Histologically, expansion of the tubule above the ligature was also striking and the lining epithelium was seen to have undergone marked change and had



Text-fig. 3*a*, *b*. Relative proportion of stained spermatozoa and decapitated spermatozoa in samples withdrawn from two separate levels of the body of the epididymis (1 and 2) following ligation in the scrotum. ——, Level 1; – – –, level 2.

become very much flattened (Pl. 2, fig. 7). In addition, distension of the seminiferous tubules with fluid and spermatozoa that were actively motile *in vitro* was conspicuous. Despite these changes, however, spermatogenesis was not obviously curtailed for at least 3 weeks (Pl. 2, fig. 6).

The incidence of stained and decapitated spermatozoa from control epididymides is shown in Text-figs. 2*a*, *b*. The trend is that of a higher proportion of degenerate

forms occurring during the 2nd and 5th weeks than in the 1st and 3rd weeks, but it seems that the response was far from being constant in all regions of the tubule. During the 2nd week, for instance, significant increases in incidence of degenerating spermatozoa were evident in samples taken from levels 1 and 3 but not from level 2 (see Table 1). It thus appears that high proportions of degenerating spermatozoa from the body of the epididymis shown during the 2nd week in Text-figs. 2*a*, *b* (where the mean of estimates from levels 1 and 2 was used for illustration) were mostly due to their presence in the distal extremity of the region, namely from level 1. This is shown in Text-figs. 3*a*, *b*, where variation in the response of spermatozoa from both parts of the body of the epididymis throughout the experiment is illustrated.

In the 3rd week, values for stained and decapitated spermatozoa showed no increase over those in the 2nd week, but in the most proximal areas (levels 3 and 4) a remarkable decline in the percentage of degenerating spermatozoa was revealed, although it was only significant as far as estimates of stained spermatozoa were concerned. At later periods this reduction was overcome and in the 5th week significant increases were again evident at all levels of the tubule (see also Table 1).

Effects of artificial cryptorchidism following ligation of the epididymis

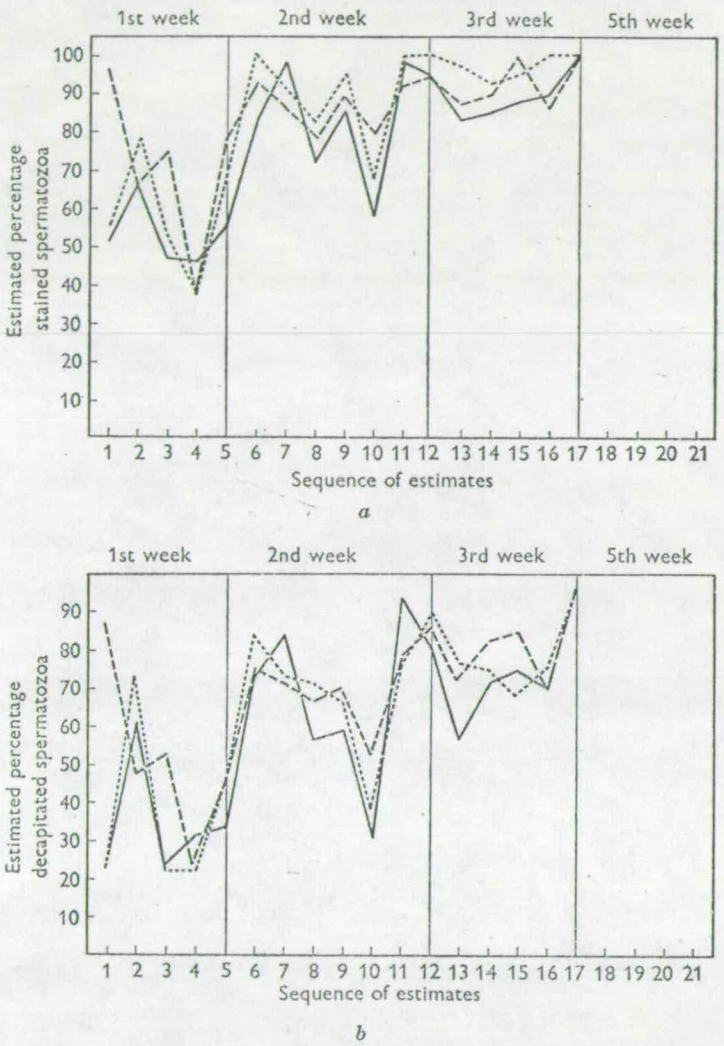
Macroscopic changes in the ductus epididymidis could also be discerned within 1 week of operation, some distension of the tubule above the level of ligation being evident, particularly in the region of the caput epididymidis. The outward appearance of the tubule, however, differed from that of control organs in that it appeared to have lost its characteristic lustre. Testes were distinctly cyanotic and when retained in the abdomen for more than 1 week they were markedly reduced in size. Disturbance of spermatogenesis was demonstrated histologically even during the 1st week and by the 3rd week complete cessation of spermatogenic activity was obvious (Pl. 2, fig. 8). Histological sections also showed the tubule of the epididymis to be distended, although in most cases the epithelium appeared to be relatively unchanged. In all animals killed during the 5th week (30–40 days) the epididymis appeared to be virtually empty of spermatozoa. Nevertheless, a sharply defined hyaline mass of fluid was evident in sections of the body of the epididymis and the head of the epididymis (Pl. 2, fig. 9).

Although during the 1st week many stained and decapitated spermatozoa were obtained from all four levels of the epididymis, their proportion increased in samples taken during the 2nd week. This effect is illustrated in Text-figs. 4*a*, *b*, and it is of interest that the incidence of eosinophilic spermatozoa and decapitated spermatozoa followed a similar pattern. Table 2 shows that increased degeneration during the 2nd week was significant in samples from the body of the epididymis and the caput epididymidis (levels 1, 2, and 3) but not from the initial segment (level 4) ($P < 0{\cdot}05$). Actually, the increases of stained spermatozoa from the distal part of the body of the epididymis and from the caput epididymidis (levels 1 and 3) were highly significant ($P < 0{\cdot}01$). No rise in mean values for either stained spermatozoa or decapitated forms was significant after the 2nd week.

In animals killed during the 5th week very few, if any, spermatozoa were discernible in samples withdrawn from levels 2, 3, and 4, although a sparse population of dis-

integrated forms was observed immediately proximal to the ligature (level 1) in two out of four specimens.

In order to assess fully the significance of these results, differences between data from cryptorchid organs and controls were next examined.



Text-fig. 4*a*, *b*. Incidence of stained spermatozoa and decapitated spermatozoa following ligation of the epididymis and retention in the abdomen. ——, Body of the epididymis (the mean of estimates from levels 1 and 2 have been used in each case); – – –, head of the epididymis (level 3); . . ., initial segment (level 4).

Differences between response of spermatozoa in cryptorchidism and in control epididymides

Although mean values in Tables 1 and 2 indicate that even during the 1st week rather more stained spermatozoa occurred in samples taken from epididymides retained in the abdomen than in those from the controls, it is important to emphasize that the differences were only significant ($P < 0{\cdot}05$) at levels 3 and 4, i.e. from the head of the epididymis and the initial segment. During the 2nd week, however,

differences at level 1 also became significant while at other levels they were highly significant ($P < 0{\cdot}01$). This means that, until the 2nd week, degeneration of spermatozoa in the body of the epididymis following artificial cryptorchidism did not differ significantly from that in the controls.

Differences between the two groups were maintained during the 3rd week and by this time differences at level 1 were also highly significant. During the 5th week a sharp increase in stained spermatozoa at all levels in the controls means that disintegration at level 1 in the two cryptorchids was not significantly greater than at the same level in the controls.

Differences in the occurrence of decapitated spermatozoa were similar to those of stained spermatozoa throughout the experiment, except that during the 2nd week they were highly significant ($P < 0{\cdot}01$) in samples taken from level 1.

These differences show that although ligation of the epididymis ultimately resulted in excessive degeneration of spermatozoa it did not obscure a distinct effect of cryptorchidism during the first 3 weeks. This was particularly conspicuous during the 3rd week owing to a reduction in degenerate spermatozoa occurring in samples from the head of the epididymis and the initial segment of the controls (Pl. 1, figs. 2 and 3).

DISCUSSION

This experiment shows that spermatozoa in the upper portions of the epididymis of rabbits are adversely affected by the conditions imposed by artificial cryptorchidism. Their response, moreover, appears to be more rapid than that reported for spermatozoa in the tail of the epididymis (Glover, 1959*b*, 1960) since degenerative changes were observed during the 1st week of retention in the abdomen. This does not necessarily mean, however, that spermatozoa in the head of the epididymis and initial segment are intrinsically the more susceptible, for their relatively early disintegration might have been due to an effect of their respective environments. Histochemical studies (Nicander, 1957) have shown that regional differences in physiological activity in the epididymis of rabbits are probable, and cytochemical findings in other species (Wislocki, 1949; Bern, 1949; Rollinson, 1955; Maneely, 1955; Cavazos, 1956; and others) suggest that variation in the activity of the lining cells might be generally characteristic of the epididymis. Earlier observations (Hammar, 1897; Priesel, 1924; Redenz, 1924; Benoit, 1926) lend further support to this contention.

Therefore, the effects of cryptorchidism on spermatozoa may be only indirect and their disintegration might not represent a response to elevated temperature *per se*. Young (1929) found that *in vitro*, spermatozoa from the head of the epididymis were less susceptible to high temperatures than those from the tail of the epididymis; this is the opposite of what might be expected from their apparent behaviour *in vivo*. Nevertheless, the present results do indicate that in the rabbit, spermatozoa in the head of the epididymis are likely to react to short periods of temperature elevation, in contrast to spermatozoa in the cauda epididymidis. If this is also characteristic of other mammalian species, it may explain the decapitation of spermatozoa in rams produced by a single 24 hr. period of insulation of the scrotum (Glover, 1955).

In the body of the epididymis, differences between data from cryptorchid and control organs suggest that spermatozoa in this region respond to the effects of

cryptorchidism in a similar way to spermatozoa from the cauda epididymidis, that is to say, they appear to show little or no morphological change during the 1st week. From this it would seem that some resistance to or protection from the effects of cryptorchidism is acquired by spermatozoa by the time they leave the head of the epididymis.

The apparent disappearance of spermatozoa from ligated epididymides during the 5th week of cryptorchidism is of interest, and since it has not hitherto been recorded, would seem to warrant further investigation. While it is conceded that spermatogenesis had at this time completely ceased, the absence of spermatozoa cannot be adequately explained as being due to migration since no outlet existed and only a few spermatozoa could be obtained from the distal extremity of the body of the epididymis, close to the point of ligation. Beyond this, no explanation can at this stage be offered for their disappearance, but whilst the highly pathological conditions involved here can be readily appreciated, it is still of interest to speculate upon whether spermatozoa are capable of disappearing from epididymides normally situated in the scrotum. The present work has shown that considerable numbers of degenerating spermatozoa can be obtained from upper regions of the epididymis following simple ligation of the tubule in the scrotum; is it possible, therefore, that dissolution of spermatozoa and absorption of disintegration products can occur in these regions of the epididymis under normal circumstances? Observations on spermatozoa from intact epididymides (Glover, 1961) suggest at least that some wastage in these regions might be normal.

Macmillan (1954) reported that ligation immediately below the head of the epididymis in rats invariably resulted in tubular rupture and spermatocele formation. These effects were not observed in rabbits in the present study, but it is to be remembered that here ligation of the tubule was performed at a lower level and this would undoubtedly allow more room for the accommodation of spermatozoa. In addition it is conceivable that the epididymis of rabbits is more distensible than that of rats and also that spermatozoa might not be produced in such profusion. However, the epididymis of rabbits does appear to adapt partially to the effects of ligation and for 3 weeks any back pressure on the testis seems insufficient to arrest spermatogenesis completely.

Adjustment of the epididymis may involve nothing more elaborate than expansion of the tubule, but present results suggest that it allows some movement of spermatozoa to continue. Otherwise a fall in the incidence of degenerate spermatozoa from the head of the epididymis and initial segment of controls during the 3rd week is difficult to explain. The results give no information, however, about the speed of migration, but doubtless movement would be slowed as a result of ligation until the 5th week when, it appears, spermatozoa might come to a halt and overall degeneration ensue. But histological evidence stresses the importance of tubular fluid in the creation of increased pressure and probably general stasis within the tubule after ligation.

It should be emphasized also that many spermatozoa that were actively motile *in vitro* were withdrawn from the testes and from all levels of the epididymis in control organs for 2–3 weeks after ligation of the tubule. This compares well with the finding of Young (1931) that following ligation of the epididymis in guinea-pigs, spermatozoa

taken from areas above the ligature showed a progressive increase in motility *in vitro* up to 25 days after the operation. In the present experiment, however, these actively motile samples contained many degenerate spermatozoa. These have also been reported in normal intact epididymides (Glover, 1961), but under conditions of epididymal ligation their progressive increase in number would seem likely to be due to interference with normal transport. Moreover, since it is probable that at any one level of the upper epididymis the total number of spermatozoa would be increased following ligation, enhanced motility of samples might be only relative to this increase and might not necessarily reflect a greater activity of individual spermatozoa.

Thus, there is no evidence in the present work to contradict the beliefs of Tournade (1913), Redenz (1924), von Lanz (1924, 1926), and Redenz & Belonoshkin (1929) that maturation of spermatozoa was due to secretions of the epididymis. On the other hand, if ligation of the ductus epididymidis does produce increased activity of individual spermatozoa, the conclusion of Young (1931) and Simeone & Young (1931), that maturation is an intrinsic property of the spermatozoa, seems to be more tenable. It may be that both factors play a part. Nevertheless, the increased incidence of degenerating spermatozoa following ligation of the epididymis indicates that more evidence is needed before enhanced motility in the samples can be regarded with certainty as representing maturation changes in individual cells.

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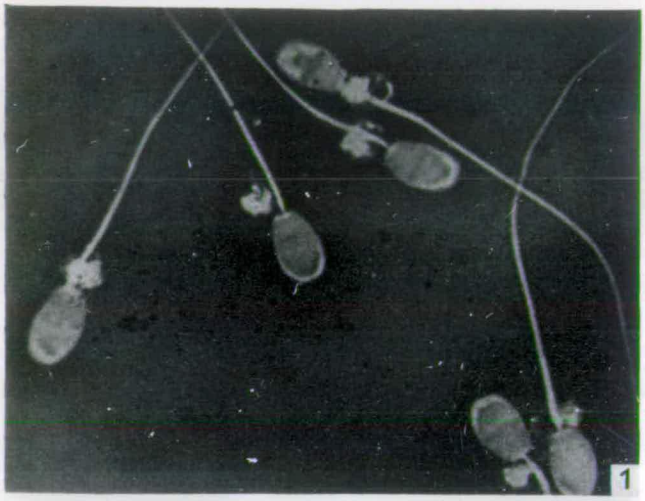
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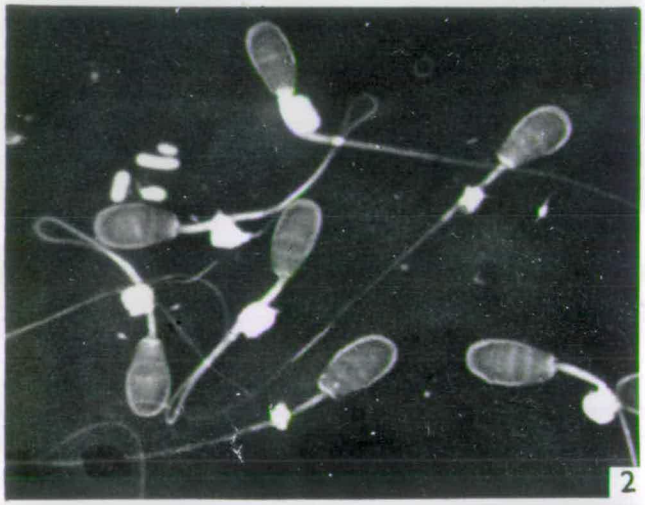
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DESCRIPTION OF PLATES

PLATE 1

Smears of spermatozoa from the head of the epididymis stained with aqueous nigrosin eosin (see p. 319). ×1500.

Comparison of Figs. 2 and 3 shows that some of the effects observed in cryptorchidism were not entirely due to ligation of the tubule.

Fig. 1. Spermatozoa from the caput epididymidis of a normal rabbit. These spermatozoa were taken from the region of the flexure of the caput epididymidis.

Fig. 2. Spermatozoa taken from the caput epididymidis 2 weeks after experimental occlusion of the tubule.

Fig. 3. Spermatozoa from the head of the epididymis 2 weeks after occlusion of the tubule and retention in the abdomen.

PLATE 2

Histological sections of testes and epididymides stained with Harris's haematoxylin and chromotrope 2*R*. ×300.

Fig. 4. Testis of a normal rabbit.

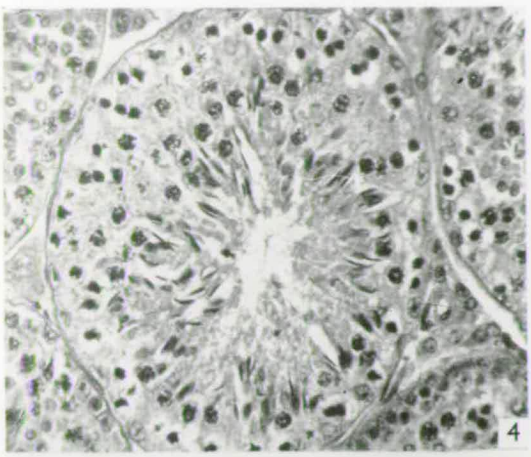
Fig. 5. Head of the epididymis of a normal rabbit.

Fig. 6. Testis of a rabbit 3 weeks after occlusion of the epididymis in the scrotum.

Fig. 7. Head of the epididymis 3 weeks after occlusion of the tubule in the scrotum.

Fig. 8. Cryptorchid testis 3 weeks after occlusion of the epididymis.

Fig. 9. Head of the epididymis 3 weeks after occlusion and retention in the abdomen.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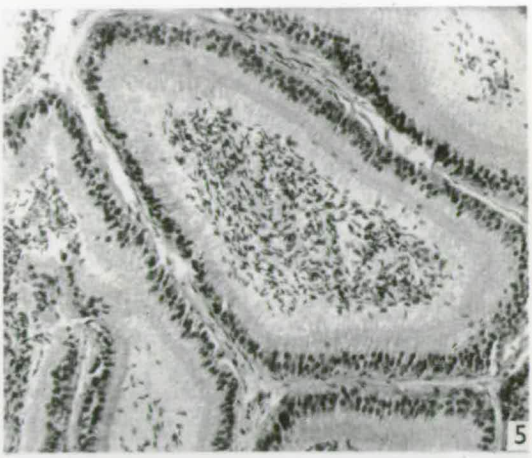
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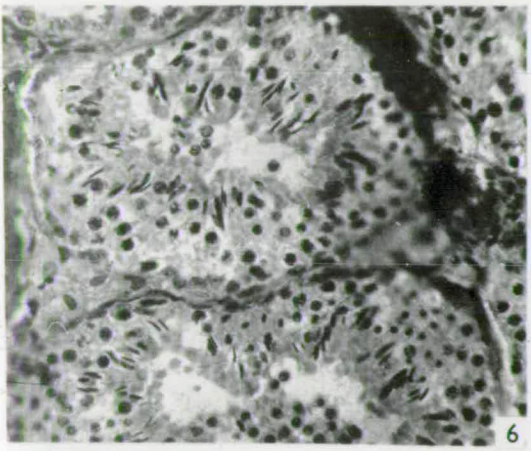
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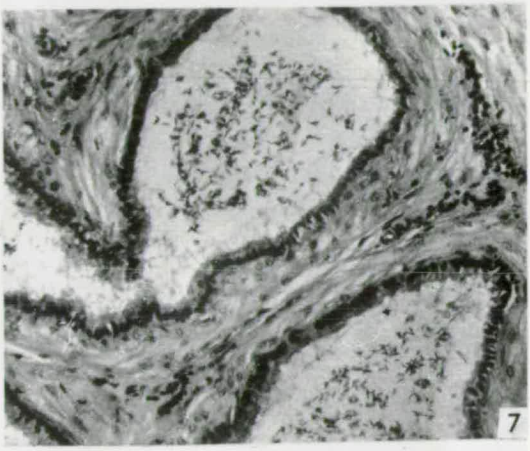
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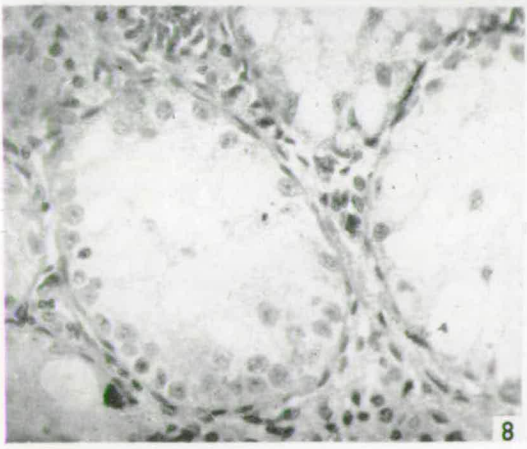
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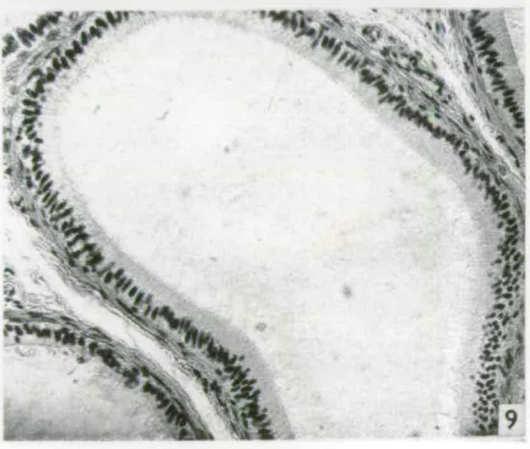
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(1967)

Temperature and the Production of Spermatozoa*

T. D. GLOVER
AND D. H. YOUNG

A number of factors well known to seminologists prevent a well-planned and clear-cut experiment from being carried out on the fertility of the human male. Largely for this reason, it would seem, a lot of data on male fertility have been obtained from experiments on animals, and many aspects of function in the human testis have necessarily involved considerable speculation. The question of temperature and its effect on male fertility is outstanding in this connection, for experimental results have been obtained on only a limited number of subjects, while other data have been derived from clinical observations where adequate control measures are often difficult to ensure. However, it is well recognized that in a wide variety of mammals, including man, the testes function at a temperature which is lower than that of the abdominal viscera. Furthermore, many experiments with animals have revealed that the production of male gametes is extremely susceptible to any increase in the temperature of the testes. It would therefore seem worthwhile to review some of the experiments and examine the situation as it applies to human problems. The present paper is an attempt to do this, and it is hoped that it might provide some perspective upon which future experiments with the human subject could be based.

Experiments on the importance of testicular temperatures

Harrison listed differences between abdominal and testicular temperatures in several mammals and pointed out that it was greatest in the rat and mouse (mean

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values being 8.3 and 8.5°C., respectively), intermediate in the rabbit and ram (6.2 and 7.1°C.) and least in the macaque monkey (2.0°C.). In man, this temperature difference is between 2.0 and 2.5°C. While the delicacy of thermoregulation in the human testis has never been firmly established, it is recognized in laboratory animals that spermatogenesis is sensitive to relatively small increases in environmental temperature.[43, 44, 50] By means of artificial cryptorchidism in guinea pigs, these workers induced testicular degeneration and established the thermoregulatory role of the scrotum. Under these circumstances the temperature of the testes would, according to Harrison's data, be raised only 4.0°C. above normal. These findings were confirmed by Moore and Oslund,[48, 49] and already similar effects had been observed in dogs by Griffiths. More direct evidence of the susceptibility of the testes to heat was provided by Fukui,[12, 13] who found that application of heat rays to the testes of rabbits resulted in defective spermatogenesis. Moore and Chase also reported this result from the direct application of heat to the scrotum.

Cunningham and Osborn sterilized rats with 5-min. treatments of infrared radiation at 48°C., and Fukui[12, 13] showed that bathing the scrotum in hot water at this temperature destroyed the spermatogenic epithelium of rabbits within 1 hr. These results are not really surprising, since physiologic mechanisms, such as enzyme systems, are likely to be impaired if not inhibited by temperatures approaching 50°C. By contrast, it appears from Fukui's work that at 40°C. very long periods of exposure are needed to produce complete degeneration of spermatogenic tissue. He gave 180–200 hr. of continuous exposure as examples. Yet Asdell and Salisbury reported damage in the rabbit testis after only 24 hr. of experimental cryptorchidism, but in comparing the two results, the degree of spermatogenic interference must be taken into account. In addition, it would seem incautious on existing evidence to regard the effects of cryptorchidism as exclusively due to increased testicular temperature. This is particularly so in clinical cases of the disorder, and this point was emphasized by Klein and Mayer. Nevertheless, it is clear that spermatogenic activity is sensitive to localized increase in temperature irrespective of its mode of induction. Guieysse-Pellisier, for example, claimed a sterilizing effect of hot steam when applied to the scrotum of dogs, and insulation of the scrotum in bulls and rams, where an increase in the temperature of the testes is unquestionable, results in characteristically deficient sperm production;[16, 24, 35, 39] similar effects have also been produced by artificial cryptorchidism in rabbits.[18] In man, subfertility is sometimes reported in association with varicocele,[57–60, 66, 67] and it has been suggested that this may be a reflection of a localized temperature effect.[26] Apparently hydroceles act similarly, and it is also likely that increased temperature plays a part in the manifestations of orchitis. In rams, for instance, Gunn *et al.* reported defective sperm production due to inflammation caused by blowfly maggot on the scrotum.

The detrimental effects of increased temperature on the function of the testes are not confined to local applications, however, for exposure of animals to high environmental temperatures also induces degeneration in the quality of ejaculated semen.[4, 39] In the human being MacLeod and Hotchkiss showed, too, that by increasing body temperature experimentally the concentration of spermatozoa in ejaculated semen could be reduced. It has been claimed also that in clinical cases involving pyrexia, the normal function of the human testis can be impaired, and pneumonia and typhoid have been mentioned in this connection.[27, 42] Further evidence of the effects of pneumonia was produced by MacLeod, who also showed that chicken pox can cause a temporary disturbance of sperm production. Gunn *et al.* demonstrated seminal degeneration in rams as a result of the intraperitoneal injection of pus from acute abscesses. The purpose of this procedure was simply to produce an experimental pyrexia.

It therefore seems clear that elevation of temperature, whether local or general, affects spermatogenic tissue adversely in mammals whose testes are normally resident in a scrotum.

Possible causes of "heat degeneration"

Fukui[12, 13] suggested that damage to the testes incurred by increased temperature was due to the thermolability of certain "spermatogenous" proteins. The contention has never been confirmed or refuted in subsequent work, but it is more often held that circulatory changes are involved. Barron believed that "heat degeneration" could be due to hyperemia resulting from vasodilatation, while Moore[44] gave vascular stagnation and oxygen lack as a possible explanation. If heat is applied to an exposed testicle, a hyperemia is apparent, as would be expected, but in the rabbit a characteristic cyanosis ensues. It would seem possible that when the temperature is elevated, blood flow to the testes might be slowed because of back pressure, and an alteration in the capillary-reduction curve might follow,[36] but Cross and Silver (1962) have shown that oxygen tension in the testis of rabbits increases when the scrotum is warmed. Circulatory factors, therefore, need more study—but certainly even slight changes in the circulation of the testes can affect spermatogenesis.[30] A relationship between circulatory and temperature effects has been demonstrated by Moule and Knapp, who showed that in the ram, ligation of the external pudendal artery causes a rise in the temperature of the testes.

An entirely different suggestion as to the mechanism of "heat degeneration" was put forward by Meschaks, who showed that seminal degeneration in bulls following insulation of the scrotum is closely related to the excretion of neutral steroids in the urine; he suggested that abnormal function of the testes under these conditions might be due to excessive production of testicular and adrenal cortical hormones.

The matter remains unsettled, but as far as the effects of circulation are concerned, quantitative data on blood flow such as those recently provided by Waites and Moule[63, 64] are the most helpful contribution. With regard to hormonal effects, consideration of the problem makes painfully clear the dearth of precise information on the endocrine control of the testes. It is perhaps for this reason that results obtained on the hormonal activity of the testes in conditions of increased temperature are difficult to interpret.

Endocrine function of the testes during "heat degeneration"

Moore and Gallagher reported that in guinea pigs 8 months of artificial cryptorchidism produced no apparent effect on the secretion of testicular hormone, and Jeffries, using the cytology of the accessory organs as an indicator, made a similar claim for the rat. Nelson,[52] however, observed changes in the seminal vesicles of rats after 240 days of experimental cryptorchidism. Moore[45] also used rats as experimental animals and took the weight of the seminal vesicles as an indicator of male-sex-hormone activity. He found that confinement of the testes in the abdomen reduced hormonal activity, although, if the condition was continued, the hormone output appeared to attain some constancy after 20–80 days. Pfeiffer showed a reduction in the size of the seminal vesicles of the rat following an increase in environmental temperature, and Elfving obtained similar results in the guinea pig with direct application of heat to the scrotum.

All these experiments relied on the weight or cytology of the accessory glands as indicators of the male sex hormone. More recently Clegg[6] used levels of seminal fructose and citric acid as indicators of male sex hormone activity in artificial cryptorchidism in rats. This was based on the work of Mann *et al.*, who had shown these levels to be accurate indicators of circulating androgen. By using these technics, Clegg was able to show that reduction in androgenic activity during cryptorchidism was preceded by a sharp rise. The finding was later confirmed[7] by means of a detailed histologic examination of the interstitial cells of the testes. Earlier, increase in seminal fructose during insulation of the scrotum in rams had been demonstrated,[17] and a similar increase in excretion of 17-ketosteroids was shown by Meschaks in bulls. This apparent increase in androgenic activity during the early stages of artificial cryptorchidism doubtless explains the findings of Bouin and Ancel, Hanes and Hooker, and others that the interstitial tissue of the testes increases in amount in cryptorchidism. Nevertheless, Engberg claimed in the human being that bilateral cryptorchidism reduced androgenic activity by half, and explanation of the cause of these fluctuations must remain speculative at present.

Possible differences in effect between naturally occurring and artificial cryptorchidism should be borne in mind, however, since in the former condition the testes have generally been exposed to an abnormal environment for a longer period of time.

The response of spermatozoa to increased temperature

Since it is necessary for spermatozoa to traverse the epididymis before they are ejaculated, it is of obvious importance to consider the effects of high temperature upon the spermatozoa themselves during their prolonged sojourn in the excurrent ducts. It might be emphasized in this regard that spermatozoa spend much of their adolescence and often their entire adult life in the epididymis. This structure is permanently packed with spermatozoa, and its length in many mammals is striking, being more than 70 M. in the stallion, at least 40 M. in bulls,[14] and roughly 6 M. in man.[62]

If spermatozoa in the epididymis were unaffected by variations in temperature, the adverse effects of cryptorchidism or insulation of the scrotum would not be revealed in ejaculated semen for some time, because the epididymis would first have to be emptied of its contents (normal spermatozoa) unless defective spermatozoa could squeeze their way through the main sperm mass. This would necessitate an extra impetus to the passage of abnormal spermatozoa, and on present evidence it would seem highly unlikely. However, during insulation of the scrotum in rams, degenerated (decapitate) spermatozoa do not appear in ejaculates for about 1–2 weeks,[16, 56] so it is relevant to ask whether this delay represents the time of migration through the epididymis. If it does, the assumption must be made that decapitation occurs only in the testes. This would not be justified for rabbits, where it has been shown that artificial crpytorchidism exerts a profound effect on spermatozoa already in the epididymis.[19, 20] In this species, however, the effect appears to vary according to the level of the epididymis at which the spermatozoa are located. Decapitation of so-called mature spermatozoa from the tail of the epididymis is not seen in artificial cryptorchidism for 7–11 days while it appears to occur more rapidly in spermatozoa situated in the head of the epididymis.[21] It thus appears that the action of increased temperature on the morphology of mature spermatozoa in the tail of the epididymis is cumulative. Nevertheless Knaus showed that rabbits become infertile after only three days of experimental cryptorchidism, and thus it seems that their fertilizing capacity is lost before decapitation occurs, and that a cumulative action of increased temperature relates only to the morphology of spermatozoa. This important possibility requires further investigation, especially since Young[68] showed that in vitro, sperms from the tail of the epididymis were most susceptible to high temperatures. This would seem to be the exact converse of the situation in vivo.

Decapitate spermatozoa can be produced in rams by a short 24-hour period of insulation of the scrotum[15] where accumulation of effect could hardly occur, but in these cases, abnormal sperms are not ejaculated for some 17–24 days, which closely resembles the time of migration of spermatozoa through the epididymis.[51] It thus seems possible that in the ram also, spermatozoa in the testes and upper regions of the epididymis react more readily to the effects of elevated temperature.

The effect of low temperature on the production of spermatozoa

Harris and Harrison demonstrated, by surrounding the exposed rat testis with a mixture of ice and salt, that application of a temperature of −5°C. for 1 hr. caused some destruction of spermatogenic epithelium. A temperature of −11°C. was needed, however, to bring about severe spermatogenic damage. These workers suggested that the effects were related to induced ischemia, presumably due to vascular spasm, and the results Cross and Silver (1962) obtained following the application of ethyl chloride to the scrotum of the rabbit appear to confirm this, since marked reduction in testicular oxygen tension was recorded. But it is again important to have some knowledge of the response of spermatozoa in the epididymis to such variations in temperature, and Chang showed that the application of ice to the scrotum of rabbits caused rapid decapitation of spermatozoa in the tail of the epididymis. Excessively low temperature therefore seems to have rapid effects on sperm production, but the effects of minor decreases in temperature need to be studied more critically.

It would seem important indeed to establish more precisely the range of temperature in which testes situated in a scrotum normally function, for the peculiar vascular pattern of the mammalian testis, the cremaster muscles, and the dartos muscle indicate in themselves that efficient thermoregulation of the testes is vital.

Conclusions

From relatively limited information on temperature and testicular function in man, together with more abundant data in animals, it is justifiable to conclude that increasing the temperature of the human testis is likely to reduce fertility. But the "population explosion" detracts from attaching too much importance to this as far as the average man is concerned. Nevertheless, it might well be profitable for the subfertile male to keep his testes "cool" for perhaps they might be especially prone to damage from high temperatures. Some reports,[9] in fact, have indicated that subfertility has been overcome by bathing the scrotum in cold water or casting away close-fitting underclothes. The evidence is, however, by no means conclusive, and a controlled experiment on the subject is strongly indicated.

A vital question today is, could a human male be *temporarily* sterilized by insulation of his scrotum, or by taking frequent hot baths? The answer would seem to be in the affirmative, provided that the insulation was adequate, the baths sufficiently frequent, and the water hot enough. An interpretation of these nebulous terms must, however, await the results of further experiments, of which some are in progress and others might usefully be undertaken in the future. Little progress can be made until more quantitative data are available and since fertility trials on the human being cannot easily be planned, particularly when background knowledge is scanty, conventional semen tests could afford valuable information as an initial approach. It would be worthwhile to

establish conclusively the effects of local applications of heat to the human scrotum on the motility, morphology, and metabolic activity of ejaculated spermatozoa.

An accurate appraisal of semen samples in mammals must take into account the differences in the structure of the male organs of reproduction in different species, as well as possible specificity in function.

Perhaps the testes of various mammalian species differ in their response to body temperature. The fact that Montremata, Cetacea, Proboscidea, Xenarthra, Hyracoidea and some of the Insectivora retain their testes permanently in the abdomen lends support to the possibility. This is particularly so where there appears to be no close correlation between body temperature and the position of the testes.[65]

That the length of the ductus epididymidis also differs between species might be of additional importance, since wide variations in the number of contained spermatozoa must occur. The time at which testicular abnormalities are revealed in ejaculated semen in different species might thus vary considerably. Figure 1 illustrates, as an example, differences in basic structure between the epididymis of the ram and that of man. Apart from over-all size differences, the tail of the epididymis of the ram is extensive when compared with that of the human being. Therefore, if spermatozoa are damaged in the testes or upper regions of the epididymis only (as, for instance, after short periods of temperature elevation), it seems likely that they might appear in human ejaculates sooner than they would in those of the ram. It could be, of course, that the response of human spermatozoa to elevated temperature is entirely different from that of

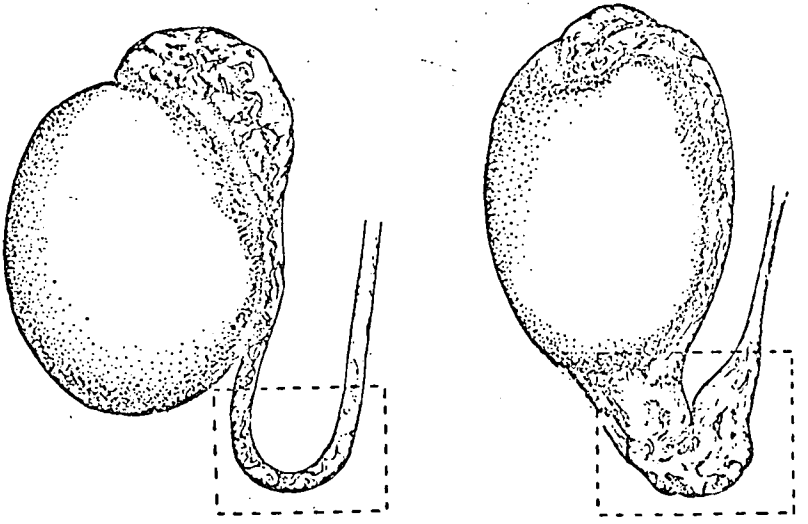

FIG. 1. Diagrams of human (*left*) and ram (*right*) testis and epididymis, illustrating form of tail of epididymis relative to rest of epididymis. (See text.)

spermatozoa in experimental animals, and also, the time of migration of spermatozoa through the human epididymis needs to be established beyond all doubt.

From this survey, we suggest that experiments on the influence of increased testicular temperature on the fertility of the human male are desirable, for it might be significant in certain cases of infertility or subfertility. In addition, application of the effects of increased testicular temperature to the problems of birth control cannot be altogether dismissed. It seems that severe heat treatment is needed in animals before the testes are damaged irreversibly, that spermatozoa in the epididymis readily respond to increased temperature by first losing their fertilizing capacity and then rapidly disintegrating. Furthermore, libido does not appear to be affected by short-term treatments. If sperm production is not completely abolished or the adverse response of spermatozoa is not total, the effects may still be of value in solving a world problem though they may be disappointing in individual cases. Certainly it seems from experiments with animals that local heat treatment of the testes is systemically innocuous, and the possibility of side effects can be disregarded. When a number of noxious agents, some of which might upset general physiologic functions, are being tested, it would seem appropriate also to investigate seriously the applicability of a well-known and apparently harmless phenomenon to the problem of population control.

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Studies on spermatozoa from the epididymis of rabbits injected with testosterone propionate following experimental cryptorchidism. By T. D. GLOVER and D. M. WOOLLEY. *Department of Veterinary Anatomy, University of Liverpool*

Whilst experiments with guinea-pigs (Moore, 1928) have indicated that in experimental cryptorchidism a decline in the motility of epididymal spermatozoa is not influenced by the presence of androgen, Elfving (1950) showed that infertility in rats, induced by the application of heat to the scrotum, could be delayed by the administration of testosterone propionate. Hitherto, however, no data appear to have been available on the effects of androgens on epididymal spermatozoa in artificially cryptorchid rabbits, and the present experiments were undertaken with a view to providing such information.

The degree of degeneration in spermatozoa was assessed by estimating the incidence of eosinophilic spermatozoa, together with that of decapitate spermatozoa following the staining of samples with nigrosin eosin differential stain (see Glover, 1960, 1962).

The first experiment involved ten adult rabbits, all of which were subjected to artificial unilateral cryptorchidism. Starting on the day of operation, five of the animals were treated with daily doses of 1 mg. testosterone propionate ('Neo Hombreol', Organon Laboratories) suspended in ethyl oleate and given subcutaneously. The other five untreated rabbits served as controls. On days 6, 8, 11, 14, and 17 one treated animal and one untreated animal were killed and spermatozoa were withdrawn from the epididymides. Differences between the percentages of stained (eosinophilic) spermatozoa from the tail of abdominally retained epididymides and those in samples from the tail of scrotal epididymides were calculated. These were 6, 13, 21, 73, and 86, on each of the above days in untreated animals, whilst in the treated group corresponding differences were 3, 12, 14, 72, and 83. Differences in the proportion of decapitate spermatozoa in the untreated animals were 2, 4, 8, 38, and 39, and in the treated animals, 1, 4, 15, 32, and 32. The results show that injections of testosterone propionate at this dosage level neither influenced the time of onset of morphological change in the spermatozoa nor significantly affected the general pattern of degeneration. Preliminary observations also indicated that degeneration in the testes was not influenced.

When the experimental procedure was repeated in a further series of rabbits following complete removal of the testes, it was again evident that degeneration of spermatozoa in abdominally retained epididymides was not delayed by testosterone propionate injections, although the incidence of disintegrated forms was considerably higher in the treated group. No significant variation in the incidence of eosinophilic or decapitate spermatozoa occurred in samples taken from epididymides that had been isolated in the scrotum, and good motility was observed up to the seventeenth day.

Androgenic effects of the injections were assessed in these animals by histological examination of the glandula vesicularis (see also Davies & Mann, 1947). Observation of the height of the epithelium in treated animals demonstrated that castration changes, such as were seen in the untreated animals, had been prevented.

P.T.O.

In conclusion, therefore, it would appear that in rabbits the presence of the testes does not influence degenerative changes in epididymal spermatozoa evoked by an abdominal environment of the epididymus. Also it would seem that these changes are not prevented or delayed by the administration of exogenous androgen regardless of the presence or absence of the testis.

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HANGES IN BLOOD FLOW IN THE TESTIS AND EPIDIDYMIS OF THE RAT FOLLOWING ARTIFICIAL CRYPTORCHIDISM

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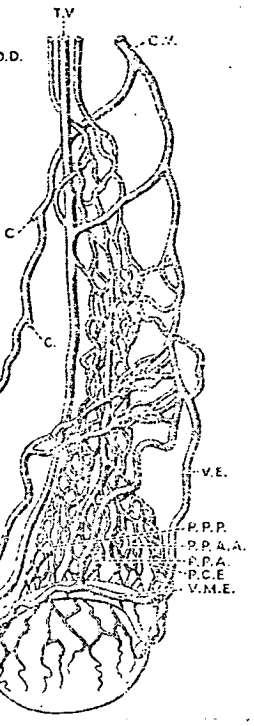
Measurements of blood flow within the testis and epididymis of rats subjected to unilateral tificial cryptorchidism were made using the method of Saperstein (1958). This method is been recently applied to an investigation of blood flow in the testis and epididymis by etchell, Waites & Till (1964). In the present work, one testis and its associated epididymis as secured in the abdomen of each rat by means of a single suture passed through the verted tunica vaginalis and the abdominal wall. Estimations of blood flow were made n groups of animals after 1 hour, 1, 2, 3, 7, 8, 10, 14 and 21 days following operation. he procedure involved anaesthetizing the animals with pentobarbitone sodium, injecting 0 μc of ^{131}I-iodoantipyrene into the femoral vein and killing within 30 seconds of the jection. Estimates of the uptake of radio-activity by the tissues were made using a scintillaon well-counter.

The rats varied in weight from 282 gm. to 358 gm., and on the control side where the gans were in the scrotum, the mean flow fraction of the testes was calculated as 0.509 % an assumed cardiac output of 205 ml/Kgm/min, whilst that of the corresponding epididymies was 0.232 %. The estimated mean blood flow in the testis was 0.317 ml/min and in the idydimis 0.071 ml/min. On a weight basis this gave a mean blood flow of 20,8 ml/100 gm/in in the testes and 13.9 ml/100 gm/min in the epididymides. With the contralateral organs at were placed in the abdomen, the mean flow fraction in the testes was 0.406 % which ve a mean total blood flow of 0.254 ml/min (24.4 ml/100gm/min) and in the epididymides e mean total flow was 0.126 ml/min (28.7 ml/100 gm/min). This reveals a difference in ood flow between the scrotal and abdominal testes but it was found to give a mean figure only 0.039 ml/gm/min. By contrast, the overall difference in blood flow between scrotal d abdominal epididymides was 0.14 ml/gm/min. In the head of the epididymides, hower, the difference was only 0.074 ml/gm/min, which indicates that the striking increase epididymal blood flow under these conditions was due to excessive increases in the lower rts of the duct. This was due particularly to a marked response in the body of the epidiymis although increased flow in the tail of the epididymis was also considerable.



n showing the venous drainage of the wed from the lateral aspect. C., *com- n between cremasteric (not shown in mpiniform plexuses.* C.V., *cremasteric ictus deferens.* D.V., *deferential vein. capitis epididymicus.* P.P.A.A., *plexus anterior accessorius.* P.P.A., *plexus anterior.* P.P.P., *plexus pampiniformis testicular vein. Venæ comitantes of this either side, and will eventually join the* E., *vena epididymica.* V.M.E., *vena idymidis.* V.M.T., *vena marginalis testis*

ally forms the testicular vein itself, is t the cauda epididymidis with a arginal vein of the testis, which ls the cauda from the mediastinum. l vein also anastomoses with the nd deferential veins and with a n of the epididymis, whose main age is into a vena epididymica at the pididymis and the deferential vein at ena epididymica drains largely into ric vein. In addition, the caput has its own special drainage by way plexus into the pampiniform plexus. nvisaged, therefore, that any venous e which exists in the cremasteric le of exerting untoward influence on s as well as the testis; it is therefore note that MacLeod (1965) has st marked changes in semen pattern, ce of varicocele, to involve sperm morphology and that the most general and striking improvements, following high ligation of the testicular vein, occur in sperm motility.

A reappraisal of the diagnosis and treatment of varicocele is urgently necessary, with particular reference to the ætiology of this condition, the treatment of which is now more rational anatomically and successful. If the surgeon is ever mindful of the eventual fertility of a patient with varicocele, the unsatisfactory results of the former classical operation of resecting the pampiniform plexus might well be avoided.

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The Influence of Temperature on Flow of Blood in the Testis and Scrotum of Rats

In 1964, Setchell, Waites & Till used the technique of Sapirstein (1958) for measuring flow of blood in the testis of the rat and ram. The method rests upon the ability of tissues to concentrate certain intravenously administered radioactive substances and to hold them at a constant level for a period of time. Setchell *et al.* (1964) showed that 4-^{131}I-iodoantipyrine was a suitable substance and already it has been utilized to assess changes in blood flow within the testis during artificial cryptorchidism (Glover 1965). However, surrounding structures, such as the tunica vaginalis and the scrotum, might normally be expected to influence the reaction of the testis to local changes in the environment; therefore, throughout the present work, which deals with local variations in temperature, the testes have been confined within the scrotum. The scrotum of different rats was exposed to several different

Table 1

Mean estimates of blood flow following thirty minutes' exposure to each temperature (The figures in brackets show the ranges of variation in estimates obtained)

		Blood flow (ml/g/min)			
Temperature	*No. of animals*	*Testis*	*Epididymis*	*Tunica vaginalis*	*Scrotum*
Controls (20° approx.)	6	20·7 (19·6–22·5)	14·1 (11·7–17·4)	9·9 (6·9–20·1)	6·4 (3·8–9·0)
−10°C	3	3·0 (0·6–5·8)	3·1 (0·8–5·7)	2·6 (0·4–6·6)	1·5 (0·3–4·5)
0°C	4	6·4 (6·0–7·7)	7·0 (3·5–11·4)	2·8 (1·5–5·5)	4·7 (1·1–8·4)
41°C	3	33·6 (25·2–40·6)	31·5 (23·4–38·6)	29·0 (13·8–49·8)	12·8 (7·2–15·7)
43°C	3	41·4 (30·3–51·5)	41·2 (32·4–55·3)	29·7 (25·2–38·8)	26·4 (19·5–37·8)

Table 2

Mean percentage increase in blood flow following exposure of scrotum to two different temperatures for different periods of time

			Mean percentage increase	
Environmental temperature	*Time (min)*	*No. of animals*	*Testis*	*Epididymis*
41°C	15	6	8	5
43°C	15	2	29	73
41°C	30	3	47	123
43°C	30	3	100	131

temperatures and estimates of blood flow were made. Changes in blood flow evoked by exposure of the scrotum to these temperatures are represented in Fig 1 and Tables 1 and 2.

Conclusions

Under the conditions of these experiments, it does seem that individual responses in blood flow were not entirely consistent, but blood flow in the scrotum of the rat responds sluggishly to variations in external temperature and that this is in contrast to events occurring in cremasteric and epididymal blood vessels. In the testis increased blood flow following warming of the scrotum is less striking than in the epididymis and this is in conformity with earlier findings in artificial cryptorchidism (Glover 1965). Nevertheless, it seems that changes in testicular blood flow are more readily achieved by warming the scrotum than by cooling it. An increase in testicular temperature of about 10°C to 12°C, brought about in these experiments by exposure of the scrotum to 41°C or 43°C for a period of thirty minutes, effected an increase in blood flow of between 50% and 100% but results suggest that it would be necessary to depress testicular temperature by at least 20°C before a 50% diminution of blood flow could be expected. This would agree with the work of MacDonald & Harrison (1954), which showed a reluctance of the testicular artery to constrict in response to cold, and does not conflict with the findings of Cross & Silver (1962) on changes in oxygen tension induced by temperature variation. Qualitatively this reaction to cold appears to apply even more to epididymal and cremasteric blood flow. Histological evidence indicates that generally the ultimate degree of testicular damage incurred by changes in external temperature can be related to more immediate changes in blood flow. However, the problem of cause and effect remains obscure for, whilst a detrimental effect of vasoconstriction is not surprising, it seems unusual for an organ to respond adversely to an increase in its blood flow *per se*. If methods other than temperature change could be used to influence blood flow in the scrotum and its contents, this problem might well be solved. Preliminary results obtained using ^{86}Rb-rubidium chloride instead of ^{131}I-iodoantipyrine confirm changes in testicular blood effected by variations in temperature, and the details of these results will be presented elsewhere.

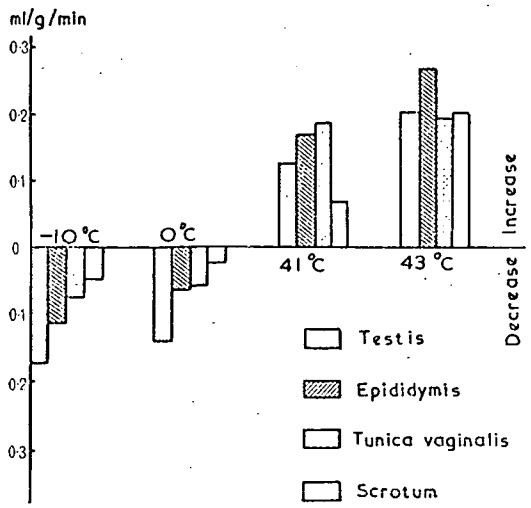

Fig 1 *Mean changes in blood flow after thirty minutes' exposure to four different temperatures*

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EFFECTS OF TEMPERATURE ON BLOOD FLOW IN THE TESTIS AND EPIDIDYMIS OF THE RAT

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Kormano (1) indicated that following heating of the scrotum of rats, a low testicular temperature was rapidly re-established. It might be expected that commensurate with this the testicular blood flow would also return to normal. Glover (2), using the method of Sapirstein (3), measured the effect of scrotal warming and of scrotal cooling on testicular and epididymal blood flow in rats, and in these experiments it was found that scrotal warming was the more effective. But the treatment was always applied for a short period (15 or 30 minutes) and there was no indication as to how long the blood flow changes persisted after removal of the source of heat. This paper, therefore, deals with testicular and epididymal blood flow both during and after scrotal warming. In addition, the relationship of testicular blood flow to temperature of the testis was determined.

MATERIALS AND METHODS. Male rats of the Sprague Dawley strain and of weight range 200-250 gm were anaesthetized with pentobarbitone sodium (Abbott Laboratories). Each rat was attached to a wire grid suspended above an insulated bath with its scrotum immersed in water at 38°C or 43°C. The blood flow of the testis, epididymis, tunica vaginalis and scrotum was

examined after 10, 15 and 30 minutes of this treatment and at 5, 10, 15, 20 and 60 minutes after a 30 minute period of bathing. In each animal 0.1 ml of 4-^{131}I-iodoantipyrine was injected into an exposed femoral vein, and the animal was killed 40 seconds later. The requisite tissues were then removed and the radioactive content of each was estimated using a scintillation well counter.

In three rats, a thermistor (Grant, Cambridge) was used to measure the intratesticular temperature during a 30 minute period, when the rats scrota were immersed in a bath at 38°C, and for a further 60 minutes after removal of the source of heat. This was repeated with the bath at 43°C. A Devices (M2R) recorder was used to record the intratesticular temperature, the rectal temperature was also measured concurrently using a telethermometer (Yellow Springs). The respirations were also counted at 5 minute intervals.

RESULTS AND CONCLUSIONS. The results show that immersion of the scrotum in a bath at 38°C has a minimal effect upon blood flow in the testis, epididymis, tunica vaginalis and scrotum, whereas a temperature of 43°C produces a marked effect. Exposure of the scrotum to 43°C or 38°C produces an immediate rise in intratesticular temperature and after 11 minutes in a bath at 43°C the intratesticular temperature equals that of the water. This is accompanied by a marked increase in testicular blood flow. By contrast, a temperature of 38°C produces a negligible effect upon testicular blood flow, although after 20 minutes, the intratesticular temperature is equal to that of the water.

Removal of the source of heat results in an immediate decrease in both temperature and blood flow in the testis, but the blood flow has not returned to normal even after 60 minutes. This is true following exposure of the scrotum to both 43°C and 38°C.

Immersion of the scrotum in water at either temperature for 30 minutes caused an increase in respiratory rate and rectal temperature, but when the source of heat was removed, both returned to normal values. It is important that the effect of this treatment on the cardiac output be determined, and an investigation of this, using Fegler's (1957) thermodilution technique, is in progress.

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Fertilizing capacity of rabbit spermatozoa following artificial cryptorchidism. By J. M. Cummins and T. D. Glover. *Department of Veterinary Anatomy, University of Liverpool*

The influence of artificial cryptorchidism on the morphology and staining characteristics of ejaculated rabbit spermatozoa has been investigated previously (Glover, 1959), and its effects on fertility have also been studied (Knaus, 1932). Hitherto, however, no attempt has been made to relate the semen picture obtained under these conditions to fertilizing capacity. The present work is an attempt to do this.

Bilateral artificial cryptorchidism was used to induce seminal degeneration in male New Zealand White rabbits, and the functional capacity of their semen was assessed from the number of cleaved eggs obtained from inseminated does. Ejaculates were collected by means of an artificial vagina (Walton, 1958); they were pooled and diluted with normal saline so as to give a constant number of spermatozoa for each insemination. Semen smears were also made after staining with aqueous nigrosin eosin. The experimental design was such that four bucks were used to provide the semen for each insemination, and having obtained control data on the bucks before operating, the effects of artificial cryptorchidism were tested by inseminating seven does/day for 7 days after operation. A daily test consisted of each doe being inseminated *per vaginam* with 25×10^6 spermatozoa in 1 ml. of normal saline. Ovulation was induced by the intravenous administration of 25 i.u. luteinizing hormone (LH) (Pregnyl, Organon Laboratories), and does were killed 48–60 hr. after insemination. In each animal, the oviducts were separated from the rest of the tract and flushed with normal saline for the recovery of eggs (see Adams, 1956). Ovaries were also examined for the number of ovulation sites. From the total number of eggs that were obtained from each doe, the percentage cleaved was taken as reflecting the fertilizing capacity of the spermatozoa. Overall loss of eggs, which was found to be about 15%, was estimated from the number of ovulation points on the ovaries, and the total number of eggs recovered from each animal.

The mean percentages of eggs cleaved in each test over the 7-day period of artificial cryptorchidism were sequentially 96, 75, 21, 21, 10, 0 and 0. This shows that there was a complete failure of fertilization after the sixth day and a serious decline in the fertilizing capacity of spermatozoa after 2 or 3 days of artificial cryptorchidism. That this was not due to a failure of sperm transport was evidenced by the presence of spermatozoa within and around the zonae pellucidae of uncleaved eggs.

As to the relationship of the semen picture to these events, a progressive increase in the incidence of eosinophilic spermatozoa, spermatozoa with coiled tails and decapitate spermatozoa, was observed in ejaculates, following artificial cryptorchidism. But the increase did not reach significant levels until several days after fertilizing capacity was lost.

The initial failure to fertilize cannot, therefore, be related to the occurrence of these particular abnormalities in permeability and morphology, but an increased proportion of spermatozoa with retained distal protoplasmic droplets (the droplet is

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usually discarded during ejaculation), occurred after 2 days of artificial cryptorchidism, and this may signify the first onset of senescent changes in the spermatozoa.

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ARTIFICIAL CRYPTORCHIDISM AND FERTILITY IN THE RABBIT

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Summary. Artificial cryptorchidism produced sterility in male rabbits within 7 days. Progressive infertility, which first appeared on the 2nd day after operation, resulted from a failure of a normal number of live motile spermatozoa to fertilize. Many such spermatozoa had not discarded their cytoplasmic droplets, and it is thought that these were heat-affected and incapable of fertilization. This is because fertilization rate and implantation rate were significantly ($P<0{\cdot}001$) correlated with the number of live (droplet-free) spermatozoa in the inseminates.

There were no adverse effects on the ability of embryos to implant or to survive gestation, and the morphology and sex ratio of foetuses at term were normal. Before fertilization failure became apparent, there were significant ($P<0{\cdot}001$) improvements in embryonic survival rates and a theory is advanced to explain these findings.

INTRODUCTION

Artificial cryptorchidism is known to cause aspermatogenesis in laboratory animals (Fukui, 1923; Moore, 1924; Lawrence, 1926; Cunningham, 1927; Mori, 1951; El Azab, 1966), and it is likely that this is primarily due to an increase in testicular temperature. The operation also has an adverse influence on the morphology and motility of epididymal and ejaculated spermatozoa, and this has been shown in the rabbit (Glover, 1958; El Azab, 1966), the rat (Mori, 1951) and the guinea-pig (Lawrence, 1926). In the rabbit, it has been reported to be accompanied by a rapid loss of fertility (Knaus, 1932; Asdell & Salisbury, 1941; Igboeli & Foote, 1967, 1969), though no detailed attempt appears to have been made to relate quantitative changes in the semen picture resulting from artificial cryptorchidism to corresponding effects on fertility. This work is an attempt to produce such a correlation and uses, as criteria of fertility, the ability of spermatozoa to reach and to fertilize ova, and the capacity of any fertilized ova to undergo normal embryogenesis.

MATERIALS AND METHODS

Experiment design

Mature, line-bred, individually caged, New Zealand White rabbits, main-

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tained on a diet of SG1 pellets (Nutrients Ltd, Liverpool) were used throughout. Forty-two adult males, each weighing over 3·5 kg, provided semen for fertility trials involving artificial insemination in a total of twenty-six groups, each of which consisted of seven virgin females. Animals that failed to ovulate were not included in the final data, which were based on 164 females only.

Preliminary data on semen quality were obtained from all males, before any fertility trials were carried out and appraisal was then continued on individual and pooled samples throughout the experiments. An initial experiment using a group of six of the bucks tested the fertility of semen ejaculated before artificial cryptorchidism ('normal' semen samples) and also on the 1st, 4th and 7th days following operation. It was found that fertility was lost by 7 days and data for the intermediate days were subsequently obtained using further groups of four to six bucks. These groups were also used to provide additional control material (see Table 1).

TABLE 1

DISTRIBUTION OF FERTILITY TRIALS

Male group (four to six) Total of forty-two animals	*Normal*		*Days after artificial cryptorchidism*													
			1		2		3		4		5		6		7	
	FR	*EV*	*FR*	*EV*	*FR*	*EV*	*FR*	*EV*	*FR*	*EV*	*FR*	*EV*	*FR*	*EV*	*FR*	*EV*
A	+	·	+	·	·	·	·	·	+	·	·	·	·	·	+	·
B	·	+	·	+	·	·	·	·	·	+	·	·	·	·	·	+
C	+	+	·	·	+	+	·	·	·	·	·	·	+	+	·	·
D	·	·	·	·	·	·	+	+	·	·	+	+	·	·	·	·
E	+	+	·	·	·	·	·	·	·	·	·	·	·	·	·	·
F	·	·	+	+	·	·	·	·	·	·	·	·	·	·	·	·
G	·	·	·	·	+	+	·	·	·	·	·	·	·	·	·	·
H	·	+	·	+	·	·	·	·	·	·	·	·	·	·	·	·
Total no. of females inseminated	21	28	14	21	14	14	7	7	7	7	7	7	7	7	7	7

+, Insemination of seven females for estimation of fertilization rate (FR) or embryonic viability (EV) (see text).

Operative procedure

Males were anaesthetized with intravenous pentobarbitone sodium (Nembutal, 60 mg/ml, Abbott Laboratories Ltd). Through a mid-ventral incision, the testes were drawn along the inguinal canals into the abdominal cavity and anchored to the abdominal wall by means of a suture taken through the inverted tunica vaginalis (Glover, 1958). Recovery was uneventful and libido appeared to be unaffected.

This operation was carried out on the same day in all the bucks of any one group.

Assessment of fertility

Semen was collected by means of an artificial vagina (Holborn Surgical Instrument Co., London) from adult males which had been selected for their high quality semen. Ejaculates were collected at 2- to 3-day intervals and after removal of any 'gel', the samples were examined at room temperature, both

individually and after pooling. Motility was scored on a subjective scale of 0 to 6, and the concentration of spermatozoa was estimated using a haemocytometer. Aliquots of the pooled semen were mixed with aqueous nigrosin–eosin for 5 min, as recommended by Campbell, Dott & Glover (1956), and then smeared on to clean glass slides. Percentages of 'live' and 'dead' (eosinophilic) spermatozoa and of different morphological types were estimated from counts of duplicate smears of each pooled sample.

Each doe in a group of seven was brought into post-pseudopregnant oestrus by means of an intravenous injection of 25 i.u. luteinizing hormone (LH, Pregnyl, Organon Laboratories), 19 to 21 days before insemination. Each was inseminated *per vaginam* with a 1-ml aliquot of pooled semen, which had been diluted to 25×10^6 spermatozoa/ml with Ringer solution (Mann, 1964), and an ovulation-inducing injection of LH was given at the same time.

Each group of females was used for an estimation of either fertilization rate (FR) or embryonic viability (EV). When possible, animals in one FR and in one EV group were inseminated at the same time with aliquots from the same pooled semen samples (see Table 1); this procedure was an attempt to reduce variations due to differences between male groups.

Fertilization rate

FR females were killed 48 to 60 hr after insemination; the numbers of ovulation points on the ovaries were recorded and each oviduct was flushed with 3 to 5 ml of Ringer solution. Ova obtained were mounted on slides under vaseline-supported cover-slips and examined under phase-contrast microscopy. The occurrence of regular cleavage was taken to indicate fertilization and, when possible, this was confirmed by observation of the nuclei of the daughter blastomeres in ova which had been fixed in acetic alcohol (Chang, 1952), stained in acetocarmine (Darlington & La Cour, 1962; Tesh, 1968) and mounted in 45% acetic acid. The presence or absence of polar bodies and of spermatozoa in the mucin layer, the zona pellucida or the perivitelline space was also noted.

Embryonic viability

Ten days after insemination, EV females were subjected to mid-ventral laparotomy under halothane (Fluothane, ICI Ltd) and nitrous oxide anaesthesia. If the animals were pregnant, the number, size and position of implantation sites were noted and pregnancy was allowed to continue. At Day 29 of pregnancy, just before term, the animals were killed and the uterine contents were examined. The position, orientation and weight of live and dead foetuses, the number of resorptions and placentae were all recorded, along with the sex of the live foetuses and any gross abnormalities. If it is assumed that the number of corpora lutea is the same as the number of ova originally released, then it is possible to estimate the implantation rate as the total number of implantation sites expressed as a percentage of the number of corpora lutea. Data on the normal development of rabbit foetuses have been given by Adams (1960a, b), and from a knowledge of the number of embryos alive at various stages of pregnancy, it is possible to estimate the survival rate of foetuses between those stages.

RESULTS

For convenience, the 'day' of the semen refers to the time elapsing between operation and the day of semen collection; thus '2-day spermatozoa' are those ejaculated by rabbits that have been artificially cryptorchid for 2 days.

Fertilization rate (*Table* 2)

The first significant decrease in FR occurred when 2-day spermatozoa were inseminated and, as the period of cryptorchidism increased, the number of eggs showing normal cleavage decreased rapidly to zero by 6 days. The first sign of infertility was a partial failure of fertilization with individual females, and total failure became apparent as early as 3 days after artificial cryptorchidism.

TABLE 2

POOLED ESTIMATES OF FERTILIZATION RATE

		Days after artificial cryptorchidism						
	Normal	1	2	3	4	5	6	7
No. of FR groups	3	2	2	1	1	1	1	1
No. of females	19†	13†	11†	7	7	6†	7	7
Ovulation points	210	153	129	88	83	72	86	59
Ova recovered	159	123	95	71	66	52	74	37
Abnormally cleaved ova	0	1	1	1	2	3	3	0
Normally cleaved ova	157	117	78	19	14	5	0	0
Fertilization rate (%)	98·7	95·1	82·1*	26·8**	21·2**	9·6**	0	0

Significance of difference from controls: * $0{\cdot}05>P>0{\cdot}001$, ** $0{\cdot}01>P$.
† One or more females failed to ovulate.

There appeared to be an increased incidence of abnormally cleaving, fertilized ova associated with insemination of deficient semen, and spermatozoa were occasionally seen around the zona pellucida of uncleaved ova as late as 6 days.

Embryonic viability (*Table* 3)

Adams (1960a, b), in a study of prenatal mortality in the rabbit, divided gestation into four main periods; namely pre-implantation, and early (up to 10 days), middle (10 to 17 days) and late (17 to 28 days) post-implantation. In the present work, however, since fertilization failure occurred so soon, data on post-implantation embryonic loss or survival was bound to be limited and, therefore, such a detailed analysis did not seem to be justified. Accordingly, the 'middle' and 'late' post-implantation periods have been pooled and are collectively called 'late'. Table 3 presents EV within the various stages of pregnancy; each estimate of survival is based on the difference between the successive sets of data, namely number of corpora lutea, total number of implantation sites, 'normal' 10-day blastocysts (which according to Adams are those of 12 mm diameter or more), and live 29-day foetuses. This method of calculation helps to allow for variations in the number of females due either to death during pregnancy or to loss of whole litters at some stage.

Preliminary analysis of the data (Cochrane, 1943) revealed that 20% to 40% of the variance that existed was binomial in nature and analysis of variance was therefore carried out on unweighted percentages of the survival rates within individual litters, converted into angles (Fisher & Yates, 1963). Variance was primarily due to the treatment of the male groups; in other words, differences in response between male and between female groups were not significant and it

TABLE 3

POOLED ESTIMATES OF EMBRYONIC VIABILITY

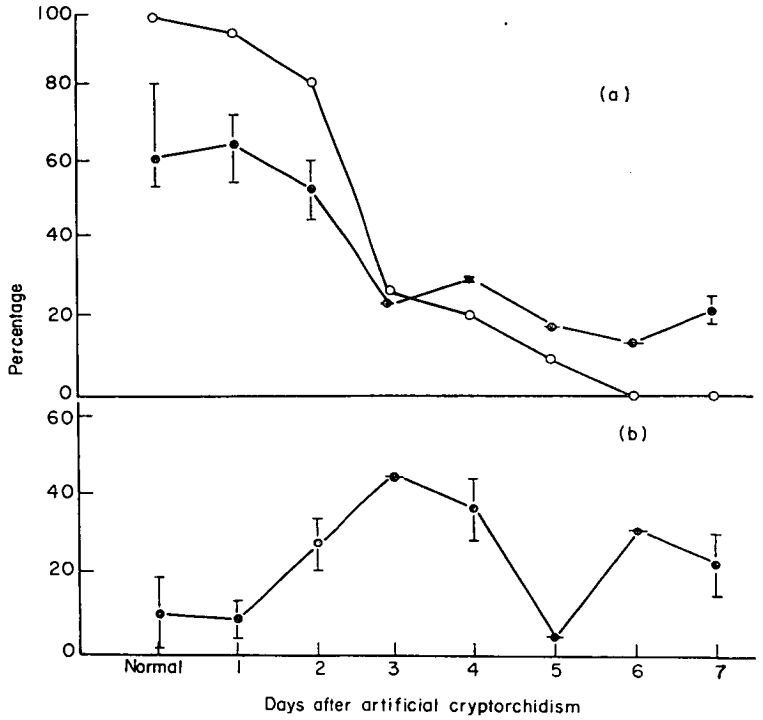
		Days after artificial cryptorchidism						
	Normal	1	2	3	4	5	6	7
No. of EV groups	4	3	2	1	1	1	1	1
Pre-implantation survival = implantation rate								
No. of females	24†	16†	13‡	6§	7	7	7	7
Total implants	196	129	129	8	40	1	2	0
Corpora lutea	260	167	149	64	95	80	85	67
Survival (%)	75·4	77·3	86·6	12·5**	42·1*	1·3**	2·4**	0**
Early embryonic survival: implantation to 10 *days*								
No. of females	23	14	23‡	3	6	1	2	0
10-day implants	172	126	113	7	38	1	1	0
Total implants	196	129	124	8	40	1	2	0
Survival (%)	87·8	97·7**	91·1	87·5	95·0	100·0	50·0	—
Late embryonic survival: 10 *to* 29 *days*								
No. of females	21‡	14	11‡	3	6	1	1	0
Live foetuses	88	111	42	3	27	0	0	0
10-day implants	166	126	99	7	38	1	1	0
Survival (%)	53·0	88·1*	42·4	42·9	71·1	0	0	—

Significance of difference from controls: * $0{\cdot}05>P>0{\cdot}01$, ** $0{\cdot}01>P$.
† One or more females failed to ovulate.
‡ Female died in pregnancy, hence eliminated from subsequent survival estimate(s).
§ Abnormal uterus, eliminated from data.

was considered justifiable to pool the data within 'days', as in Table 3. A complete analysis of variance is not included in these results because the data present such a striking picture, and where in Table 3, degrees of significance of difference from the normal data are indicated, they have been calculated on ranked percentages for individual litters using the Mann-Whitney non-parametric U-test, corrected where necessary for tied data (Goldstein, 1964).

It is apparent from Table 3 that infertility in the EV females was mainly due to implantation failure and that there were no adverse effects on EV. On the

contrary, when Day-1 semen was tested, there were significant and consistent improvements in both early and late survival rates, as compared to the normals. This resulted in a significantly higher ($P<0{\cdot}001$) mean litter size at term, of $8{\cdot}07\pm1{\cdot}69$ compared to $4{\cdot}63\pm2{\cdot}41$. Control litter sizes were smaller than those reported by Adams (1960a, b) and this apparent improvement in fertility might be regarded as spurious. But analysis of variance of the data in the four control EV groups, as well as in the three 1-day EV groups, revealed no significant within-day variations and it must be concluded that the evidence for increased fertility on Day 1 is statistically sound. It is also interesting that Day-2 semen produced no change in the implantation rate, despite the fact that the FR in females inseminated with the same semen (Tables 1 and 2) was significantly reduced. This might be interpreted cautiously as representing a decrease in the number of fertilized ova lost before implantation.

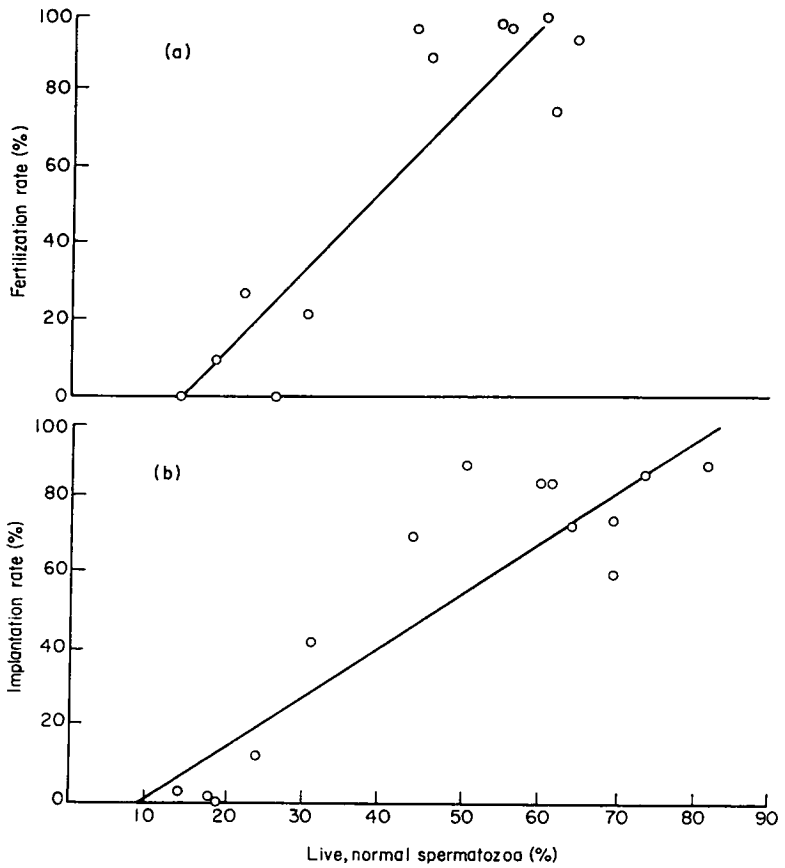


TEXT-FIG. 1. (a) Percentage of live, normal spermatozoa (●); mean and range of estimates on pooled samples of semen. ○, FR. (b) Percentage of live spermatozoa with retained cytoplasmic droplets; mean and range of estimates on pooled samples of semen.

In all, 133 live, normal, male foetuses were recorded out of a total of 247 (48·54%), and chi-squared analysis failed to show any significant within-day variations from a 50:50 sex ratio. The overall mean foetal weight was $43{\cdot}54\pm7{\cdot}20$ g and variations between litters were such that no meaningful trends could be detected. There was a negative correlation between the number of foetuses in a litter and the mean weight of those foetuses. However, unlike the observations of Hammond (1934) and Tesh (1968, 1969), this was not statistically

significant ($r = -0{\cdot}0136$, 45 d.f.). The orientation of live foetuses at term appeared to be random; 43% faced towards, and 57% away from the cervix.

No teratogenic effects could be detected resulting from the insemination of semen from artificially cryptorchid rabbits; thirteen foetuses were found to have abnormalities of the parietal bones. However, these occurred in control as well as in experimental EV groups and were all offspring of the same male group (C, see Table 1).



TEXT-FIG. 2. (a) Regression of FR upon the percentage of live normal spermatozoa inseminated. o, mean for one FR group of females. $r = 0{\cdot}9055$, N = 12, $P<0{\cdot}001$, b y/x = 2·1250. (b) Regression of implantation rate upon the percentage of live, normal spermatozoa inseminated. o, mean for one EV group of females. $r = 0{\cdot}8602$, N = 14, $P<0{\cdot}001$, b y/x = 1·3184.

Number of ovulations

Analysis of variance of the numbers of ovulation points or corpora lutea per female revealed no significant variations between any of the experimental groups. This means that either criterion can equally well be taken to represent the number of ova released and also that a certain amount of direct comparison between the estimates of FR and EV is justified. The mean number of ovulation sites or corpora lutea was $11{\cdot}26 \pm 2{\cdot}73$ (N=164).

Semen characteristics

The first quantitative indication of change in semen quality occurred on Day 2, with an increase in the proportion of live spermatozoa with retained distal (end of mid-piece) cytoplasmic droplets (Text-fig. 1b and Pl. 1, Fig. 1). These forms were invariably motile and did not shed the droplet even after several hours *in vitro*; the droplet frequently appeared to be expanded and tended to distort the mid-piece of the spermatozoon. Normally, the cytoplasmic droplet is discarded from the end of the mid-piece at ejaculation and, in fact, the best indicator of changing fertility was found to be the incidence of live, morphologically normal, droplet-free spermatozoa (Text-fig. 1a). Text-fig. 2 shows two scatter diagrams of FR and implantation rate respectively, plotted against the percentage of live, morphologically normal spermatozoa used for each group of females. Although the spread of the data tends to exaggerate the closeness of the correlations, they are both highly significant and similar to one another in slope of regression ($P<0{\cdot}001$); this means that changes in implantation rate associated with changes in semen quality closely paralleled changes in FR.

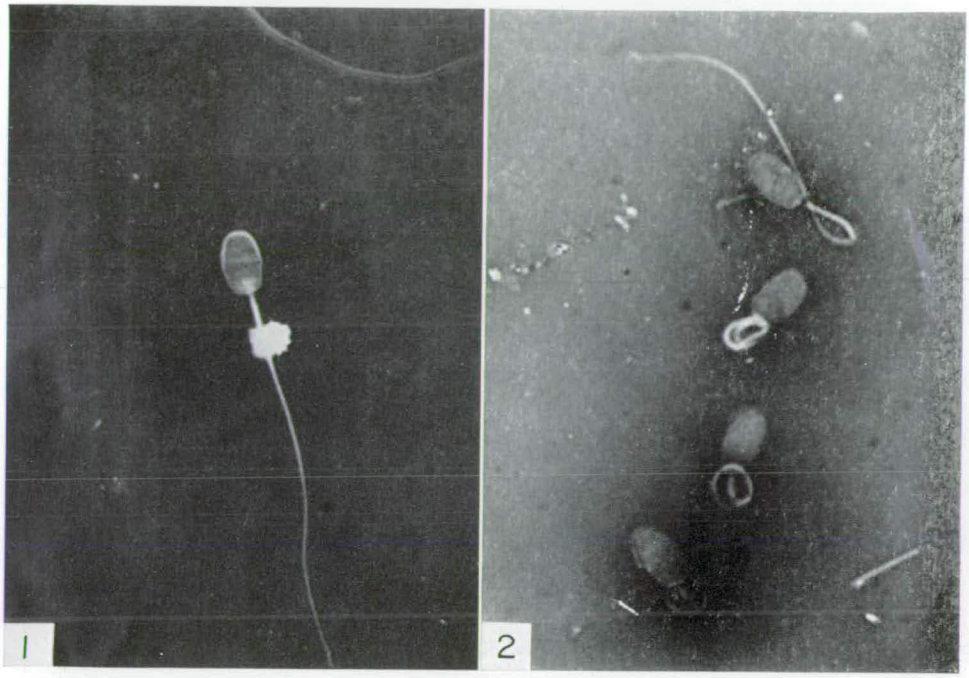
The overall live–dead ratios in nigrosin–eosin smears and estimations of the motility of spermatozoa showed a gradual decline in the quality of ejaculates over the 7-day period of study. However, these criteria could not be directly related to loss of fertility. On Day 7, for example, when the animals were completely sterile, ejaculates still contained a mean of 43% live cells, and were accredited a mean motility score of 3. On the other hand, increased percentages of abnormal spermatozoa, such as those with coiled tails and fragmented forms (Pl. 1, Fig. 2), did not occur until the 6th or 7th day.

DISCUSSION

This work has shown that rapid and progressive infertility resulting from artificial cryptorchidism appears primarily as a failure in fertilization. The onset of infertility is not due to any change in the incidence of live motile spermatozoa, although there is a highly significant correlation between FR and a reduced proportion of live, 'normal' spermatozoa in inseminates. The term 'normal' is used here with reference to those actively motile spermatozoa that have discarded the cytoplasmic droplet. Probably, therefore, live spermatozoa that retain the droplet *in vitro* not only indicate incipient seminal degeneration but may themselves be 'senescent' types with reduced, or lost, fertilizing competence.

It is well recognized that, in the mammalian epididymis, the cytoplasmic droplet migrates along the mid-piece of a spermatozoon during the process of so-called sperm maturation. The droplet is normally discarded during ejaculation and this suggests that its function, if indeed it serves one at all, concerns the life of a spermatozoon only within the male tract. This is supported by the identification of lysosomal enzymes in the cytoplasmic droplets of ram and bull spermatozoa, for there has been speculation that shedding of the droplet may be important in enabling the gamete to survive in a free state (Dott & Dingle, 1968). The present results are the first experimental demonstration of a definite association between the failure of some spermatozoa to discard their cytoplasmic droplets and a distinct deficiency in seminal function. At present, it is impossible

PLATE 1



Nigrosin–eosin smears, × 1000.

FIG. 1. Eosinophobic, live spermatozoon with distal cytoplasmic droplet; 2 days of artificial cryptorchidism.

FIG. 2. Eosinophilic, dead spermatozoa with coiled tails; 8 days of artificial cryptorchidism.

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J. Reprod. Fertil. (1963) **5**, 13-22

EFFECTS OF ALKYLATING AGENTS ON SPERMATOGENESIS IN THE RABBIT

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(*Received* 26*th June* 1962)

Summary. Serial sperm counts have been made in rabbits treated with alkylating agents. After single injections of Busulphan, Tretamine and isopropyl methanesulphonate, aspermia developed during the 10th and 11th weeks. Other simple monofunctional esters of methane and ethane sulphonic acids, which induced sterility in rats as a result of their action on spermatozoa and late spermatids, did not depress the sperm count or interfere with sperm motility in the rabbit. The results suggest that the duration of spermatogenesis in the rabbit is about 10 weeks and indicate that in this species, as in rats and mice, a steady turnover of spermatozoa occurs. Fertility tests in male rabbits after administration of Tretamine showed that small doses readily induced sterility which could be maintained by continued administration.

INTRODUCTION

It has been shown that a number of alkylating agents which may or may not be capable of inhibiting experimental tumours can produce selective effects on spermatogenesis (Jackson, Fox & Craig, 1959, 1961). Thus in male rats and mice serial weekly matings after treatment reveal characteristic phases of sterility which are related to the affected phase of spermatogenesis (Jackson *et al.*, 1959, 1961). The sterility may be due to selective damage to early germ cells or to an action on spermatids or morphologically mature spermatozoa in the testis and epididymis. In the former case, the sperm content of vaginal smears found after mating with the treated males indicated oligospermia or aspermia. Where interference with late phases of spermatogenesis is involved, the vaginal smears after insemination appeared to contain the usual abundance of motile spermatozoa. In the latter circumstances, there was no definite evidence whether or not the number or morphology of spermatozoa had been affected. It was largely for this reason that experiments with rabbits were undertaken.

MATERIALS AND METHODS

EXPERIMENTAL ANIMALS

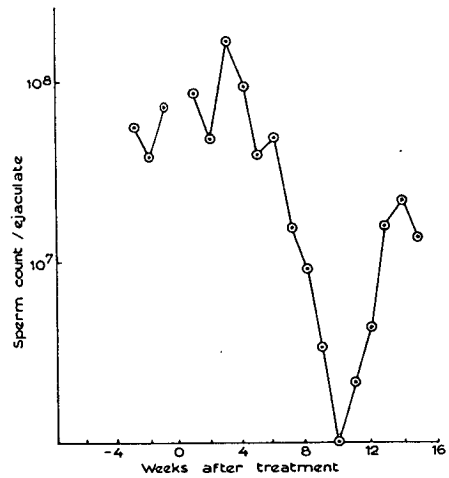
The rabbits used were of Dutch and English breeds, 2 to 3 kg in weight and maintained on standard SG1 rabbit and guinea-pig pellets (Mill Hill formulation) with water *ad libitum*. The diet was supplemented once weekly with fresh carrots or cabbage.

COMPOUNDS

Five drugs have so far been examined, namely, Tretamine (N N′ N″ triethylenemelamine) (TEM), Busulphan (Myleran, 1,4 dimethanesulphonoxybutane) methyl ethanesulphonate ($C_2H_5.SO_2.OCH_3$) (MES), methyl methanesulphonate ($CH_3.SO_2.OCH_3$) (MMS) and isopropyl methanesulphonate ($CH_3.SO_2.OCH(CH_3)_2$) (IMS). It would be desirable to correlate the sperm count with fertility in rabbits, but because of the technical problems involved only fertility results with Tretamine are so far available.

COLLECTION OF SEMEN

Ejaculated semen was collected by means of an artificial vagina (Macirone & Walton, 1938; Walton, 1958) with a modification described by Glover (1959). Specimens were obtained regularly twice a week. Only rabbits providing control values for at least 6 weeks before treatment were used. These values were usually in the range 5 to 25×10^7 spermatozoa per ejaculate, with a volume varying between 0·10 and 0·90 ml. The gel accompanying the sperm-



TEXT-FIG. 1. Mean weekly sperm count per ejaculate after treatment with a single dose of TEM (0·2 mg/kg intravenously in saline solution). The development of aspermia in the 10th week is seen, corresponding probably to an action of the drug on spermatogonia.

atozoa in the ejaculates of some rabbits could be removed with forceps and did not contain a significant number of spermatozoa. An aliquot of 0·1 ml was taken from each sample and diluted to 20 ml with approximately 0·5% formol-saline solution. Counts were made in the Fuchs-Rosenthal haemocytometer. The morphology of fresh spermatozoa was examined using the nigrosin-eosin differential stain (Glover, 1960).

FERTILITY TEST

Bucks of proven fertility were used. They were treated with various dose levels of TEM and were mated consecutively once weekly with does, also of proven fertility. Since relatively large numbers of females of proven fertility were

TABLE 1

EFFECT OF TEM ON THE FERTILITY OF MALE RABBITS

Dose (mg/kg)	*Duration of treatment (days)*		*Period No. and, in parentheses, range (days)*												
			1 (0 *to* 7)	2 (8 *to* 14)	3 (15 *to* 21)	4 (22 *to* 28)	5 (29 *to* 35)	6 (36 *to* 42)	7 (43 *to* 49)	8 (50 *to* 56)	9 (57 *to* 63)	10 (64 *to* 70)	12 (78 *to* 84)	15 (99 *to* 105)	16 (106 *to* 112)
1 × 0·2	1	a	3/4	1/3	0/2	1/2	2/2	1/1	–	–	–	–	–	–	–
		b	2·0	0·7	0	1·0	3·5	7·0	–	–	–	–	–	–	–
7 × 0·05	7	a	1/3	–	0/3	1/1	2/3	1/1	–	–	–	–	–	–	–
		b	0·7	–	0	2·0	3·0	5·0	–	–	–	–	–	–	–
10 × 0·05	10	a	–	–	–	0/2	0/2	2/2	2/2	1/2	1/2	–	–	–	–
		b	–	–	–	0	0	4·5	3·0	3·0	3·5	–	–	–	–
29 × 0·05	56	a	2/2	0/2	0/2	–	–	–	–	–	0/2*	0/2*	0/2*	0/2*	0/2*
		b	3·0	0	0	–	–	–	–	–	0	0	0	0	0
36 × 0·01	70	a	2/2	2/2	1/2	0/2	0/2	–	–	0/1	–	0/1	0/1	–	0/1
		b	8·0	2·5	1·5	0	0	–	–	0	–	0	0	–	0
29 × 0·01	56	a	1/1	–	0/1	–	0/1	1/1	–	–	–	1/1	–	–	–
		b	6·0	–	0	–	0	1·0	–	–	–	8·0	–	–	–

(a) Number producing litters out of number inseminated. (b) Average litter size. * Aspermic ejaculates.

required for these experiments, it was found necessary to use females again, after an interval of not less than 2 months.

RESULTS

TRETAMINE

A single intravenous dose (0·2 mg/kg) in saline solution had no effect for 7 weeks, after which the sperm count progressively declined to aspermia in the 10th week (Text-fig. 1). Recovery commenced in Week 11 and had approached normal levels by the 13th week. Fertility tests indicated that a sterile period occurred in the 3rd week, with a period of subfertility before and after the infertile stage (Table 1). There was, however, no alteration in sperm count

TABLE 2

TOTAL PERCENTAGE SPERMATOZOAL ABNORMALITIES IN RABBIT EJACULATES AFTER TREATMENT

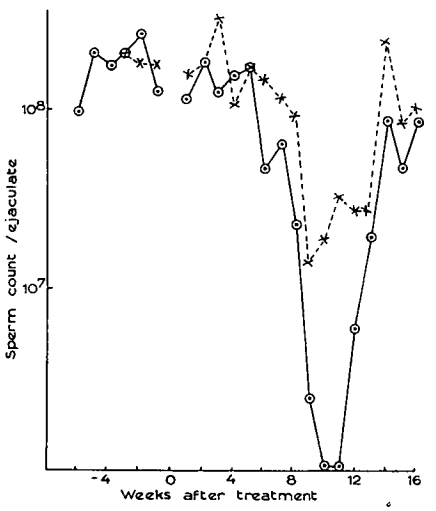
Period (*days from treatment*)	TEM	MMS	MES	IMS
−14 to −10	8	10	7	5
−9 to −5	6	9	17	5
−4 to 0	8	9	14	6
1 to 4		3	8	8
5 to 9	7	13	7	
10 to 14	8			7
15 to 19	7	14	16	5
20 to 24		17	12	
25 to 29	9	35	11	11
30 to 34	13			12
35 to 39		33	57	10
40 to 44	37	12	28	
45 to 49	22			33
50 to 54	22	30	42	26
55 to 59		22	46	
60 to 64	36	22	27	28
65 to 69	34			34
70 to 74		7	26	37

The total percentages of primary and secondary abnormalities after administration of alkylating agent are shown in this Table. These abnormalities are considered (Blom, 1950) to be due to defects occurring within the testes and epididymides, respectively. The incidence of spermatozoa with coiled tails has not been recorded because it is difficult to determine what proportion of them is due to artifacts (Bishop, Campbell, Hancock & Walton, 1954).

during this time, nor were there any changes in the motility or morphology of spermatozoa (Table 2) which could be correlated with the loss of fertility. There was some evidence, however, of an increased incidence of abnormal spermatozoa from the 40th day onwards.

The routine determination of fertility in the rabbit is more difficult than in the rat. Owing to restricted accommodation, it was only possible to obtain data from a limited number of animals.

A series of additional fertility tests was undertaken to examine the sensitivity of rabbit spermatogenesis to multiple doses of Tretamine. Seven daily doses of 0·05 mg/kg intravenously produced temporary sterility in the three treated animals during the 3rd week (Table 1). After ten such doses, both animals were mated from the 4th week onwards. They became sterile within Weeks 4 and 5 of being treated, but later recovered their fertility. More prolonged treatment (twenty-nine doses, 0·05 mg/kg each; *see* Table 1) led to infertility from the 2nd week of mating onwards. Sterility was maintained for the remainder of the experiment (duration 16 weeks). Spermatozoa were found in the vagina after each mating until the 9th week when none was seen. During this latter period, semen collected in the artificial vagina was of average volume, had a superficial cloudy appearance, but contained no spermatozoa. No effect on the *libido* of either male was noted throughout the experiment. The cumulative action was tested at a lower dose level (thirty-six doses of 0·01 mg/kg intravenously) and again sterility occurred in both males for at least 20 weeks. A shorter course (twenty-nine doses of 0·01 mg/kg) in a single animal, resulted in sterility from the 3rd week of treatment onwards. This animal subsequently recovered its fertility (Table 1).



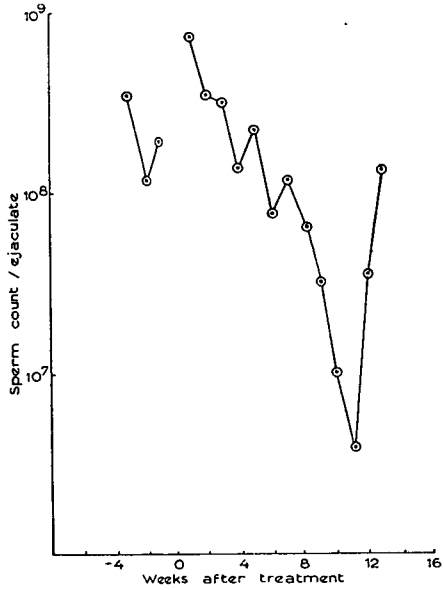
TEXT-FIG. 2. Mean weekly sperm count per ejaculate in the rabbit after treatment with single doses of Busulphan: - - - - - 10 mg/kg, ——— 40 mg/kg; given intraperitoneally in Arachis oil. A depression of sperm count occurs between Weeks 9 and 12, with aspermic ejaculates in Weeks 10 and 11 at the higher dose, correlating with an action of the drug on early phases of spermatogenesis.

BUSULPHAN

The disulphonic ester was administered intraperitoneally as a finely divided suspension in Arachis oil. A single dose of 10 mg/kg caused a depression of the spermatozoal count during Weeks 9 to 14 after treatment (Text-fig. 2), whilst 40 mg/kg produced mean values of less than 10^6 spermatozoa per ejaculate in Weeks 10 and 11. There was no change in either *libido* or volume of seminal fluid during the whole period of the experiment.

ISOPROPYL METHANESULPHONATE

This compound was administered intravenously (100 mg/kg) as a freshly prepared solution (5·8 ml) in Sorenson's phosphate buffer (pH 7·7). This procedure was followed because of the relative instability of the compound in aqueous solution (Ross, 1962, quotes a half-life of 13 min at 37° C) with the production of the strong methane sulphonic acid. The number of spermatozoa



TEXT-FIG. 3. Mean weekly sperm count per ejaculate after treatment with a single dose of IMS (100 mg/kg intravenously in phosphate buffer, pH 7·7). A minimum count occurred during the 10th to 11th week corresponding to an action on early stages of spermatogonial development.

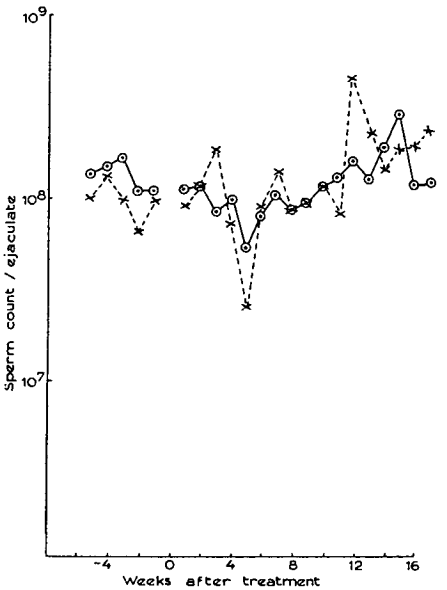
began to fall in Week 7 with a minimum value occurring during Week 11 and being followed by a rapid recovery to normal levels (Text-fig. 3). A significant increase in the incidence of abnormal spermatozoa was observed from 45 days onwards.

METHYL ETHANESULPHONATE AND METHYL METHANESULPHONATE

Neither of these substances in doses close to the maximum tolerated dose produced any significant alteration in sperm count, nor any radical change in the morphology of spermatozoa during the first few weeks after treatment (Text-fig. 4). The results support the view of Jackson *et al.* (1959, 1961) that the sterility observed in rats after these substances is not due to oligospermia or the production of morphologically abnormal spermatozoa. Also, they provide additional evidence for the suggestion that these substances do not interfere with spermatogonial development. There was again some evidence in later weeks of an increased incidence of morphological abnormalities in spermatozoa, especially after treatment with MES.

DISCUSSION

The assessment of fertility by systematic matings in the rat after treatment with alkylating agents (Bock & Jackson, 1957; Jackson *et al.*, 1959, 1961) does not adequately distinguish sterility due to oligospermia from that due to other effects on spermatozoa. It is thus desirable to obtain quantitative data regarding



TEXT-FIG. 4. Mean weekly sperm count per ejaculate in a rabbit after treatment with MES (50 mg/kg intravenously in saline solution). No significant changes occurred in the count. The graph also demonstrated the constancy of the sperm count taken over relatively long periods.

× - - - - - × MMS (30 mg/kg).
⊙ ——— ⊙ MES (50 mg/kg).

the concentration and morphology of spermatozoa after treatment. A satisfactory technique for the collection of ejaculates from rats or mice under relatively normal physiological conditions is not available, but the technique used in the present experiments for rabbits gave consistent and reliable results. Systematic data on the fertility of rabbits is difficult to obtain, and thus any correlation made between the sperm count of this species and that of the fertility of rats, is largely based on accumulating evidence that the time sequence of spermatogenesis and epididymal transport in the two species is of similar duration, i.e. 9 to 10 weeks (Heller, 1948).

Tretamine caused a depletion of spermatozoa in the ejaculates of rabbits 10 weeks after treatment (Text-fig. 2), which is comparable with the observation (Steinberger, Nelson, Boccabella & Dixon, 1959) that a transient depletion of spermatogonia occurs in the rat after treatment with the same compound. Furthermore, the subfertility observed in experiments with rats (Jackson *et al.*, 1961) 9 to 10 weeks after treatment could be accounted for by a similar depletion. There was neither change in sperm morphology nor diminution in the

sperm count of the rabbit in the 4th week, when sterility evidently occurs both in this species and in the rat. It is thus apparent that the sterility is most likely to be due to some inconspicuous change in the spermatozoa. The term 'dominant lethal mutation' has been used to describe the cause of this TEM-induced sterility in mice (Cattenach & Edwards, 1958), on the assumption that genetic material within the spermatozoa has been damaged by the alkylating agent.

In order to test the susceptibility of rabbits to the antifertility action of TEM several protracted courses of treatment have been tried in an attempt to determine the minimal dose required to induce and maintain sterility (Table 1). The lowest dose used in our experiments (0·01 mg/kg intravenously daily), produced sterility by the 4th week, and this persisted throughout the experiment. Lower dose levels were not attempted, but clearly, these doses did not represent the minimum that could be expected to induce an effect.

Busulphan selectively inhibits early spermatogonial development in the rat (Jackson *et al.*, 1961) and sterility develops after a delay which corresponds to the duration of spermatogenesis (9 to 10 weeks). In the rabbit, oligospermia did not occur until 10 weeks after treatment (Text-fig. 2), and this time was probably a reflection of the overall duration of spermatogenesis. Histological sections of both rat and rabbit testis, 24 days after treatment with Busulphan, show a similar state of depletion (Plate 1). These results support the view that the timing of spermatogenesis in the two species is similar.

The action of the branched-chain monofunctional compound, IMS (Text-fig. 3) resembles that of Busulphan and TEM where early stages in spermatogenesis are also affected. There is some evidence of increased morphological abnormalities in spermatozoa during the 6th to 7th week but without diminution in count or less of motility. In the rat, it has also been found that infertile spermatozoa appear about the 6th week.

The simple straight-chain esters, MES and MMS, however, produced no change in the total numbers of spermatozoa in rabbit ejaculates (Text-fig. 4). There is as yet no supporting data on the fertility of the rabbit, but comparable doses to groups of rats caused sterility in the 2nd and 3rd weeks following treatment. The increased incidence of abnormal spermatozoa in rabbit semen, observed from the 6th week onwards, suggests that some action on earlier stages of spermatogenesis may have occurred. The normal spermatozoal count during Weeks 10 to 12 after treatment supports the view that spermatogonial phases are not readily susceptible to interference by these straight-chain esters.

ACKNOWLEDGMENT

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PLATE 1 OVERLEAF

EXPLANATION OF PLATE 1

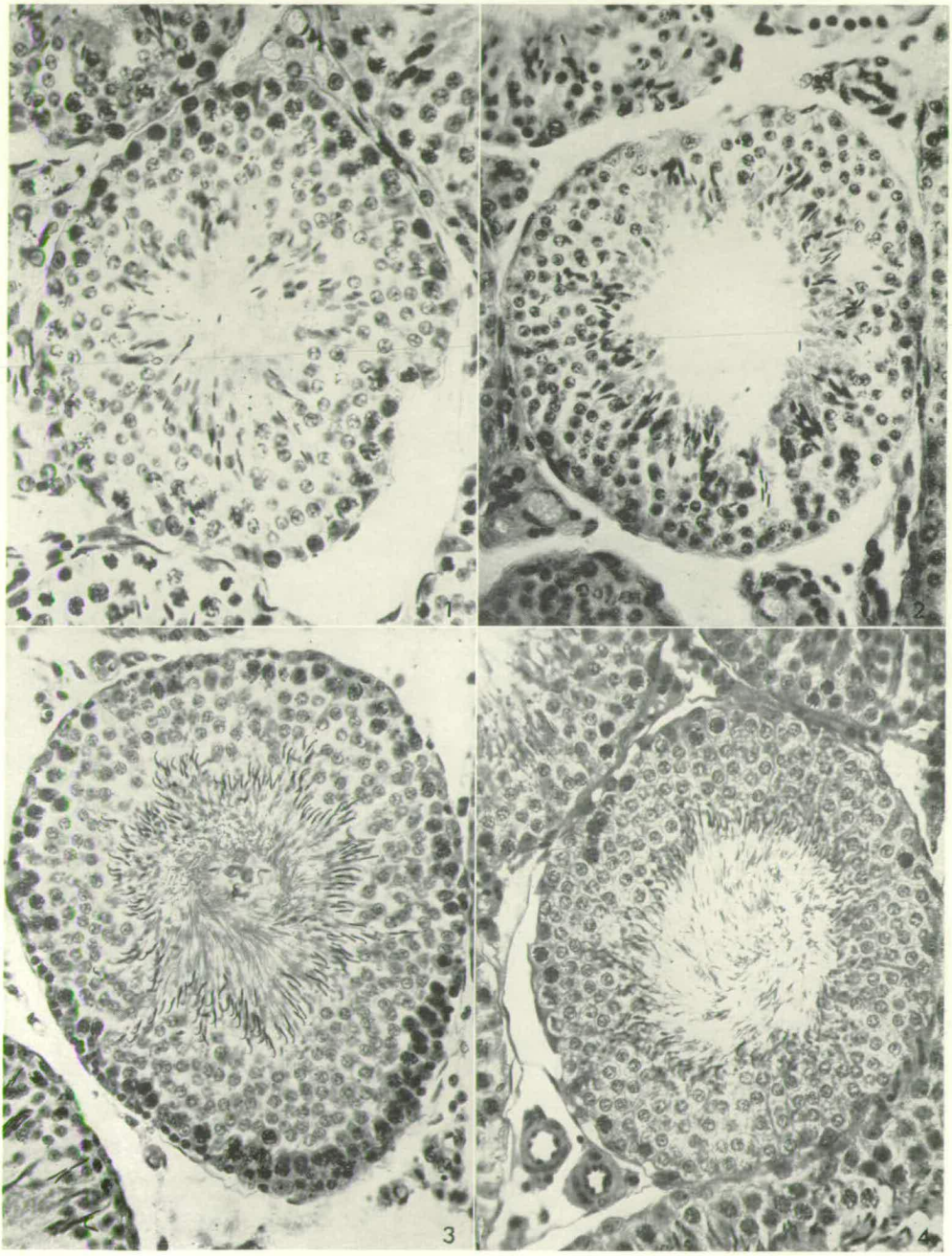
FIG. 1. Section of normal rabbit testis tubule.

FIG. 2. Twenty-four days after treatment of rabbit with 40 mg/kg Busulphan showing almost complete absence of spermatogonia and pachytene spermatocytes.

FIG. 3. Section of normal rat testis tubule.

FIG. 4. Twenty-four days after treatment of rat with 10 mg/kg Busulphan. A state of depletion comparable to that in Fig. 2 indicates that the maturation depletion is occurring at a similar rate in the two species.

PLATE 1



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J. Reprod. Fertil. (1965) **9**, 119–130

SOME REACTIONS OF RABBIT SPERMATOZOA TO LIGATION OF THE EPIDIDYMIS

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Summary. Experiments have been carried out to investigate the reaction of epididymal spermatozoa to interruption of their normal passage through the epididymis. In eighteen rabbits, the epididymis was ligated unilaterally at the distal end of the body, and in a further fifteen the ligature was placed at a point just distal to the head of the epididymis. Samples of spermatozoa from different regions proximal to the ligature were examined in each case for changes in motility, morphology and reactions to certain staining techniques.

It was found that ligation resulted in a distinct pattern of changes in the contained spermatozoa, irrespective of the level at which the epididymis was ligated. Initially these changes involved an enhanced capacity for motility *in vitro*, decreased eosinophilia and an increased number of coiled tails. In addition, maturation changes in the acrosome and in the location of the protoplasmic droplet occurred. After 14 days of ligation, however, degeneration of the spermatozoa was widespread.

The significance of these events is discussed in relation to the normal maturation of spermatozoa. It is suggested that spermatozoa can mature *in situ* when the epididymis is ligated, but that the changed environment resulting from ligation appears to superimpose a number of morphological abnormalities on the spermatozoa.

INTRODUCTION

The motility *in vitro* of mammalian spermatozoa removed from the testis or upper levels of the epididymis is poor compared with that shown by spermatozoa removed from the tail of the epididymis. The increase of motility during the passage of spermatozoa along the ductus epididymidis is widely accepted as reflecting a process of maturation, and it would appear that it is accompanied by an enhanced ability of the spermatozoa to fertilize.

However, Young (1931) showed in guinea-pigs that, if the epididymis is ligated, spermatozoa above the ligature become actively motile *in vitro*. Thus it seems possible that, in this species at least, spermatozoa can 'mature' at any level of the epididymis, and Young concluded that maturation is primarily a temporal phenomenon. This is not in accordance with the view of Tournade (1913), Redenz (1924), or von Lanz (1926) that secretions of the epididymis are the most potent influence in this process. Salisbury (1956) suggested, as a

compromise, that both intrinsic and extrinsic factors might be involved, but the issue was far from being settled and the need for further investigation was clear.

The work to be described approaches the problem of maturation in spermatozoa from the point of view of their morphology and certain staining characteristics. The rationale for this lies in the fact that the spermatozoon is also subject to changes in its structure and permeability as it passes through the epididymis, and these, too, have been regarded as indicators of maturation. Migration of the protoplasmic droplet from the neck of the spermatozoon to the end of the mid-piece is a well-known change, and alteration in the shape and dimensions of the acrosome have also been recorded in the rabbit by Bedford (1963), and in the guinea-pig by Fawcett & Hollenberg (1963). In addition, peculiar changes in the permeability of spermatozoa to eosin seem to occur during their transit through the epididymis (Brochart & Debatène, 1953; Ortavant, 1953; Glover, 1962a, b), and a conclusive explanation of these effects has never been given.

The maturation of spermatozoa is therefore not easy to define, and it was thought that the use of epididymal ligation would further contribute to an understanding of the problem.

MATERIALS AND METHODS

Thirty-three adult male rabbits of mixed strain were used in two main experiments. In eighteen rabbits, the epididymis of one side was ligated close to the distal end of the corpus epididymidis (at B, Text-fig. 1), whilst the contra-lateral organ was left intact to serve as a control. These animals will be referred to hereafter as 'the low ligation group'. The operative procedure previously described (Glover, 1960) was followed, but in this instance a single suture was used and no portion of the epididymis was removed.

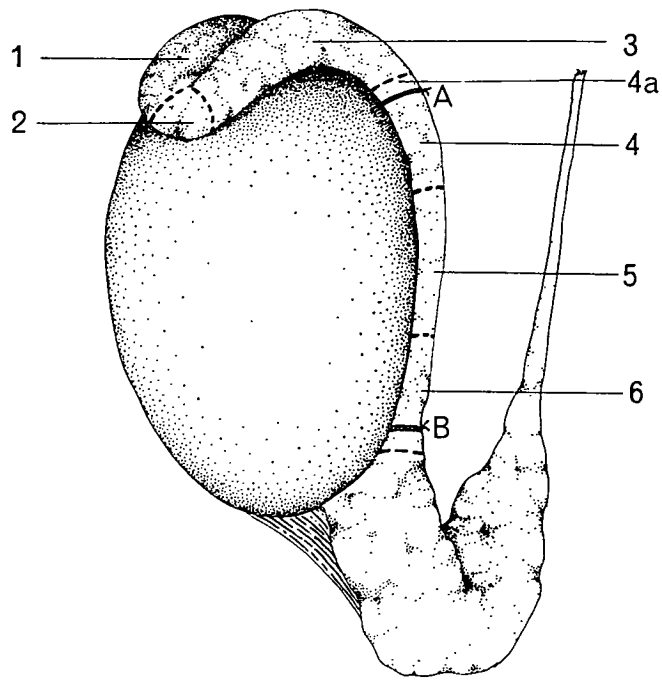
In the other fifteen rabbits, unilateral ligation of the epididymis was again performed but the ligature was this time passed round the upper end of the corpus epididymidis (at A, Text-fig. 1). These rabbits constitute the 'high ligation group'. It was considered important to have the two groups for comparison, since spermatozoa that are immediately stopped by high ligation (at point A) will be younger than those just proximal to a ligature placed at B. Moreover, it is possible that environmental conditions differ at these two levels (*see* Nicander, 1957).

It is pointed out, in addition, that of the control epididymides twelve were selected at random to provide quantitative estimates, and these have been taken together to serve as control data for both experimental groups (*see* Tables 1, 2 and 3).

Bilateral low ligation of the epididymis was effected on three additional rabbits in order to check some of the more equivocal findings and to determine whether any of the effects of ligation occurred before 6 days. Data from these animals are not included in the main analyses.

Animals in the low ligation group were killed in groups of six on the 6th, 10th and 14th days after operation, whilst five of the high ligation group were killed at the same intervals.

The rabbits were killed by intravenous injections of sodium pentabarbitone (Euthatal, May and Baker Ltd.) and immediately after death the testes and epididymides were examined macroscopically before being put in normal saline. Within 5 min the contents of a ligated epididymis were sampled at various levels above the ligature (*see* Text-fig. 1) by making a small incision in the duct and placing one drop of the emergent fluid on to each of two microscope slides. On one, the sample was smeared in the fresh state, then fixed in air and stained by a method based on that of Papanicolaou (1942); the other was smeared after



TEXT-FIG. 1. Diagram of the rabbit testis and epididymis to show alternative sites of ligation and regions from which spermatozoa were withdrawn. A, site of high ligation; B, site of low ligation; 1, initial segment; 2, caput flexure; 3, caput epididymidis; 4, upper part of corpus epididymidis; 4a, lowest level from which sperms were taken following high ligation; 5, middle part of corpus epididymidis; 6, lower part of corpus epididymidis.

being mixed with a drop of aqueous nigrosin eosin, and was then allowed to dry in air at room temperature. The motility of spermatozoa in samples from each level of the epididymis was assessed qualitatively from simple microscopic observation.

Counts of 100 spermatozoa were made on each nigrosin eosin smear at a magnification of 400, and estimates of the incidence of eosinophilic spermatozoa were made together with the estimated occurrence of certain morphological characteristics. These included coiled tails, proximal protoplasmic droplets (situated at the neck of the spermatozoon) distal protoplasmic droplets (migrated towards the end of the mid-piece) and morphologically abnormal droplets. Characteristics of the acrosome were estimated only on smears stained by Papanicolaou's stain, when 100 spermatozoa per slide were counted under oil

immersion, since these characteristics are less easily defined in nigrosin eosin smears.

All control material was examined in the same way as the experimental material, but counts of acrosome characteristics were made on samples from eight animals only.

For convenience, Tables 1 and 3 only include the results of counts from the initial segment, the caput epididymidis and the region just proximal to the ligature. Counts from levels 2 and 3 have been combined, and those from levels 4 and 5 in the low ligation group have been omitted since they did not show any important individual differences in the particular features recorded in these two tables.

RESULTS

Control material

Examination of the contents of control epididymides confirmed earlier reports that more eosinophilic and decapitate spermatozoa occur in samples taken from the lower part of the body of the epididymis than in those taken from either the tail or the head (Glover, 1962a). Also the motility of samples could be roughly correlated with the content of unstained spermatozoa, so that spermatozoa from the tail or head of the epididymis showed greater motility than those from the initial segment or lower part of the corpus epididymidis.

Counts of proximal and distal protoplasmic droplets revealed that in the rabbit the droplet migrates to the end of the mid-piece as the spermatozoa pass from the initial segment (level 1, *see* Text-fig. 1), through the caput flexure (level 2) to the head of the epididymis (level 3). Thus sperm samples from the head of the epididymis contain abundant spermatozoa with migrated droplets. Nevertheless, these spermatozoa show much less propensity for motility *in vitro* than spermatozoa from the tail of the epididymis, and in any case are accompanied by many immotile eosinophilic spermatozoa.

From smears stained by the Papanicolaou method, there appeared to be four types of spermatozoa distinguishable by their acrosomes:

(1) The acrosome was closely applied to the nucleus and formed an almost continuous outline with the postnuclear cap (*see* Pl. 1, Fig. 4).

(2) The acrosome was expanded but had a distinct outline (*see* Pl. 1, Fig. 3).

(3) Spermatozoa with a 'swollen' and very pale-staining acrosome, the outline of which was quite indistinct (*see* Pl. 1, Fig. 3).

(4) Spermatozoa that appeared to have no acrosome.

Quantitative estimates of these different types of spermatozoa showed that in samples from the tail of the epididymis the ratio of type 1 to type 2 was 94 : 5, whereas in those from the head of the epididymis it was 2 : 78. Type 4 occurred but rarely, whilst type 3 was more common in samples withdrawn from upper regions of the epididymis than in those from the tail (*see* Table 4).

Low ligation group

Overall distension of the epididymis proximal to the point of ligation occurred in all cases. However, as previously reported (Glover, 1962a, b) there were discrete patches where the tubule was particularly distended and cream in

TABLE 1

THE MEAN INCIDENCE (WITH STANDARD ERRORS) OF EOSINOPHILIC SPERMATOZOA AND SPERMATOZOA WITH COILED TAILS FOLLOWING LIGATION OF THE EPIDIDYMIS

	Initial segment (level 1)		*Caput epididymidis (levels 2 and 3)*		*Region proximal to ligature (level 6 in low ligation group) (level 4a in high ligation group)*	
	Eosinophilic (%)	*Coiled tails* (%)	*Eosinophilic* (%)	*Coiled tails* (%)	*Eosinophilic* (%)	*Coiled tails* (%)
Low ligation						
Controls	28·40 ± 4·69	7·30 ± 3·23	34·50 ± 3·09	2·50 ± 0·82	49·90 ± 7·73	1·80 ± 0·26
6 days	30·83 ± 7·89	1·17 ± 0·83	23·50 ± 4·09–*	9·75 ± 5·83	14·83 ± 2·75–**	36·17 ± 7·96**
10 days	12·50 ± 4·86–*	18·33 ± 11·95	13·75 ± 4·11–**	49·00 ± 9·88**	26·00 ± 10·45	39·83 ± 9·63**
14 days	47·67 ± 6·92*	17·33 ± 8·20	27·91 ± 4·91	10·08 ± 4·18	42·33 ± 15·45	14·50 ± 4·84*
High ligation						
Controls	28·40 ± 4·69	7·30 ± 3·23	34·50 ± 3·09	2·50 ± 0·82	30·33 ± 6·53	1·91 ± 0·98
6 days	35·67 ± 14·34	17·00 ± 16·34	18·87 ± 3·86–**	25·90 ± 12·22	22·25 ± 6·38	35·60 ± 14·38*
10 days	10·20 ± 2·65–**	45·00 ± 9·72**	21·50 ± 6·37	33·60 ± 7·33**	27·25 ± 4·77	39·75 ± 13·21*
14 days	21·50 ± 0·29	67·75 ± 13·55**	30·44 ± 5·94	35·78 ± 6·59**	69·40 ± 3·82**	24·40 ± 11·21

* Significant difference ($0·01 < P < 0·05$) between corresponding figure and that for controls (–* where difference is one of reduction).
** Highly significant ($P < 0·01$) (–** where difference is one of reduction).

colour. After 6 days of ligation, the whole of the corpus epididymidis was distended in this way, whilst the head and initial segment were unaffected. Longer periods of ligation, however, resulted in a variable distribution of these patches (for example, *see* Pl. 2, Fig. 1). They were evidently due to excessive accumulations of spermatozoa.

After 6 days the motility of spermatozoa from all three levels of the body of the epididymis was better than those of control samples, but after 10 days it was from the head of the epididymis and the initial segment that the most actively motile spermatozoa were obtained. After 14 days there was an overall reduction in the motility of the samples.

TABLE 2

THE MEAN PROPORTION (WITH STANDARD ERRORS) OF SPERMATOZOA WITH PROXIMAL DROPLETS AND DISTAL DROPLETS IN THE INITIAL SEGMENT (LEVEL 1)

	Low ligation		*High ligation*	
	Proximal (%)	*Distal* (%)	*Proximal* (%)	*Distal* (%)
Controls	58·10 ± 3·29	0·90 ± 0·60	58·10 ± 3·29	0·90 ± 0·60
6 days	50·17 ± 9·82	4·83 ± 3·57	41·50 ± 21·43	4·25 ± 1·97
10 days	40·83 ± 13·40	8·17 ± 4·81	17·60 ± 7·13–**	13·40 ± 5·71*
14 days	14·83 ± 7·15–**	9·33 ± 3·42*	9·75 ± 5·59–**	4·75 ± 2·95

* Significant difference ($0{\cdot}01 < P < 0{\cdot}05$) between corresponding figure and that for controls (–* where difference is one of reduction).

** Highly significant difference ($P < 0{\cdot}01$) (–** where difference is one of reduction).

Changes in motility were directly related to the incidence of spermatozoa with coiled tails but inversely related to the incidence of eosinophilic spermatozoa. Table 1 shows that the proportion of spermatozoa with coiled tails rose significantly at level 6 after 6 days of ligation, and at levels 2 and 3 after 10 days of ligation; after 14 days the proportions were reduced at all levels of the epididymis. By contrast, the incidence of eosinophilic spermatozoa was significantly reduced at level 6 after 6 days, and in the more proximal regions after 10 days. It appears, therefore, that ligation resulted first in the spermatozoa acquiring an increased capacity for motility *in vitro*; this was reflected in nigrosin eosin smears by a higher incidence of unstained spermatozoa and many spermatozoa with coiled tails. It was followed by the progressive degeneration of spermatozoa, the onset of which was indicated by an increased proportion of eosinophilic spermatozoa in samples withdrawn after 14 days of ligation.

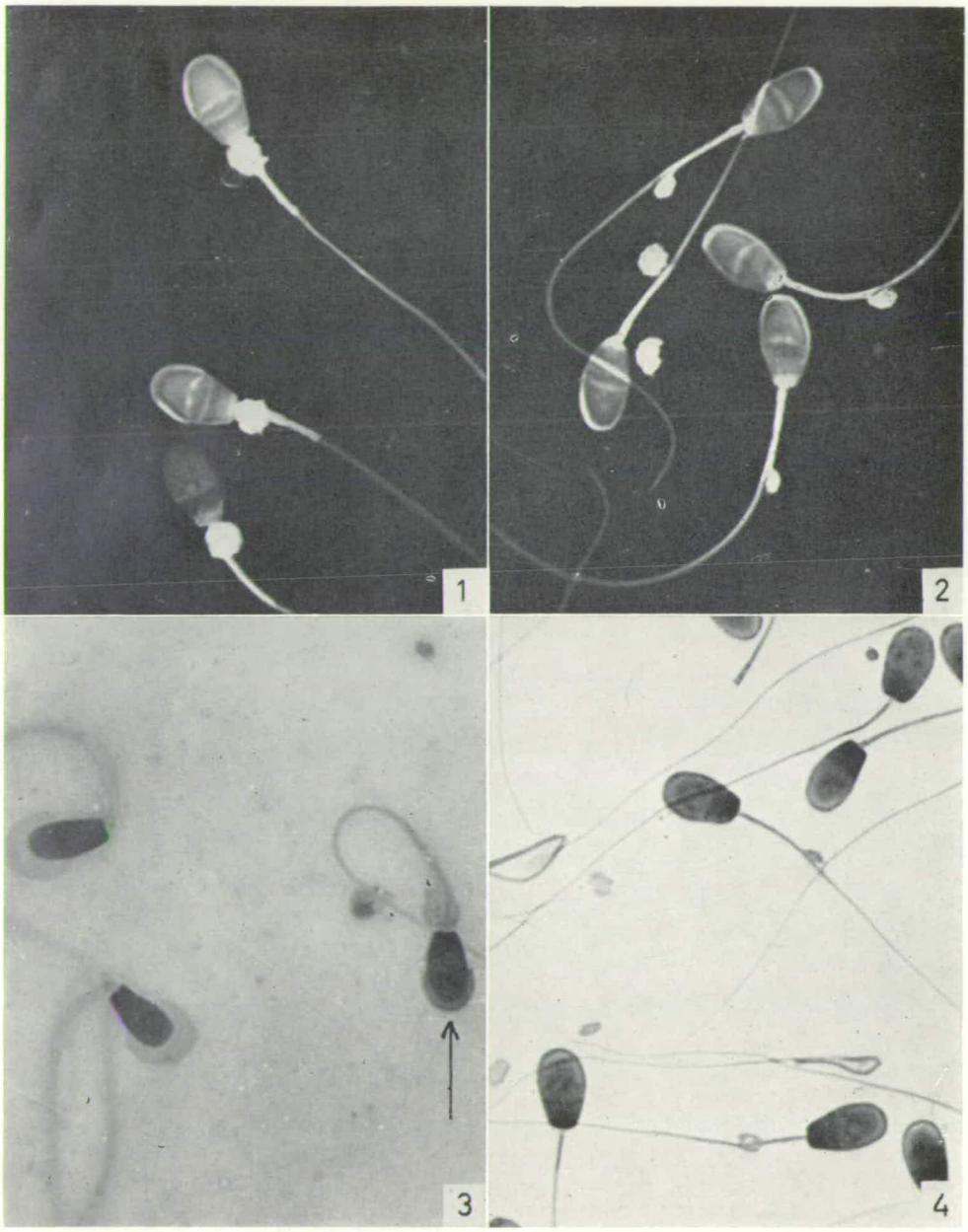
With regard to the migration of the protoplasmic droplet, results showed that in samples from level 1 the proportion of spermatozoa with proximal

EXPLANATION OF PLATE 1

Smears of spermatozoa from a control epididymis. ×1500.

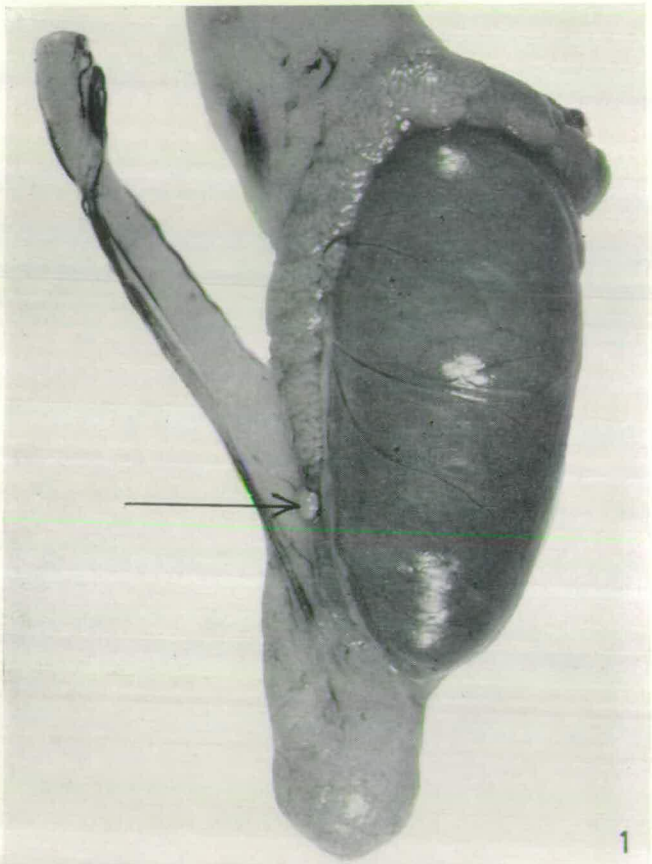
FIG. 1. Spermatozoa from the initial segment stained with aqueous nigrosin eosin.
FIG. 2. Spermatozoa from the tail of the epididymis stained with aqueous nigrosin eosin.
FIG. 3. Spermatozoa from the caput flexure region stained by Papanicolaou's technique. The photograph shows one spermatozoon with type 2 acrosome (arrowed, considered to be immature) and two spermatozoa with type 3 acrosomes (considered to be dead) for comparison.
FIG. 4. Spermatozoa from the tail of the epididymis stained by Papanicolaou's technique.

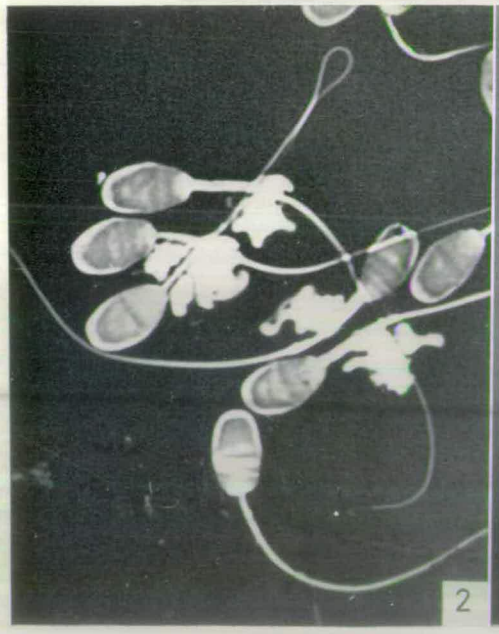
PLATE 1

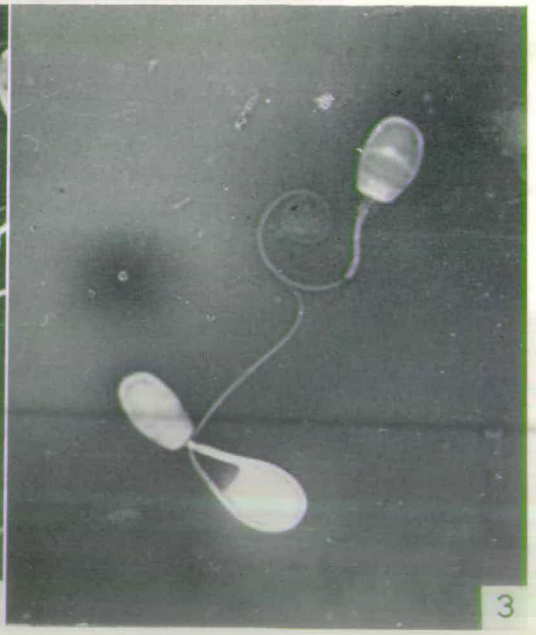


(*Facing p.* 124)

PLATE 2







droplets was significantly reduced after 14 days, but an increased incidence of distal droplets was less significant (*see* Table 2). In addition, some of the spermatozoa from all levels had morphologically abnormal droplets after 6 days of ligation and these took the form of swollen or fragmenting droplets (*see* Pl. 2, Figs. 2 and 3). There was considerable variation between individuals in the proportion of these abnormalities, but a highly significant increase in their occurrence was apparent after 14 days in samples from the head of the epididymis (*see* Table 3). It might be noted in passing that swollen droplets were frequently associated with coiled tails (*see* Pl. 2, Fig. 2).

TABLE 3

THE MEAN INCIDENCE (WITH STANDARD ERRORS) OF SPERMATOZOA WITH ABNORMAL PROTOPLASMIC DROPLETS

	Initial segment (*level* 1)	*Caput epididymidis* (*levels* 2 *and* 3)	*Region proximal to ligature* (*level* 6 *in low ligation*) (*level* 4*a in high ligation*)
Low ligation			
Controls	0·00	0·92 ± 0·42	1·10 ± 1·10
6 days	2·00 ± 0·73	3·58 ± 1·55	5·50 ± 2·51
10 days	7·67 ± 3·92	5·75 ± 2·52	4·67 ± 3·03
14 days	10·33 ± 5·15	15·66 ± 1·25**	6·33 ± 3·35
High ligation			
Controls	0·00	0·92 ± 0·42	0·33 ± 0·24
6 days	2·75 ± 2·14	1·80 ± 0·66	9·00 ± 6·61
10 days	2·60 ± 1·44	19·80 ± 4·14**	9·50 ± 2·72**
14 days	3·00 ± 1·78	20·12 ± 8·09*	2·80 ± 0·86*

* Significant difference ($0{\cdot}01 < P < 0{\cdot}05$) between corresponding figure and that for controls.
** Highly significant difference ($P < 0{\cdot}01$).

Differential counts of acrosome types in samples taken from level 2 showed that low ligation of the epididymis eventually results in a significant increase in this area of the proportion of spermatozoa with type 1 acrosomes. In two out of the three animals examined after 3 days of ligation, the percentage frequency of type 1 acrosomes was 68 and 88 respectively, whilst in the third animal no significant increase in this type of acrosome was discernible. After 6 days, a highly significant increase in the occurrence of type 1 acrosomes was apparent at this level in four out of five experimental animals, and at 10 and 14 days some stability appeared to have been reached (*see* Table 4). A high percentage of type 1 acrosomes was accompanied by a correspondingly low incidence of type 2. The proportion of type 3 acrosomes progressively decreased after ligation, but this trend only became significant after 10 days.

EXPLANATION OF PLATE 2

FIG. 1. Appearance of the testis and epididymis after 10 days of low epididymal ligation. Note marked distension of tubule proximal to the ligature. Conspicuous convolutions throughout the body and in part of the head. Arrow indicates site of ligation.

FIG. 2. Spermatozoa from the caput epididymidis following epididymal ligation showing typical fragmentation of the protoplasmic droplet (stained with aqueous nigrosin eosin). ×1500.

FIG. 3. Spermatozoa from the caput epididymidis following epididymal ligation showing typical coiling of the tail. Note one spermatozoon shows another type of droplet abnormality [*see* text]. (Stained with aqueous nigrosin eosin). ×1500.

High ligation group

Generally speaking, the effects of high ligation of the epididymis resembled those of low ligation, but differences in degree and time of onset of the changes are of interest. Overall distension of the epididymis proximal to the ligature was again apparent, with discrete creamy-coloured patches occurring randomly. High ligation often caused swelling of the seminiferous tubules also, so that white patches appeared on the surface of the testis.

The spermatozoa removed from level 4a (*see* Text-fig. 1) were the first to show an enhanced motility, whilst spermatozoa from more proximal levels did

TABLE 4

THE MEAN INCIDENCE (WITH STANDARD ERRORS) OF SPERMATOZOA WITH DIFFERENT TYPES OF ACROSOME AT THE CAPUT FLEXURE FOLLOWING LIGATION OF THE EPIDIDYMIS. CONTROL DATA FROM BOTH THE CAPUT FLEXURE AND THE CAUDA EPIDIDYMIDIS ARE ALSO SHOWN FOR COMPARISON

	No. samples	*Type* 1†	*Type* 2	*Type* 3	*Type* 4
Controls (Caput flexure)	8	2·42 ± 2·21	77·71 ± 5·48	17·00 ± 5·09	2·85 ± 0·83
Low ligation					
3 days	3	56·00 ± 21·63*	34·00 ± 23·26	7·67 ± 1·20	2·33 ± 1·45
6 days	5	71·20 ± 18·11**	18·40 ± 14·44–**	8·00 ± 3·89	2·40 ± 1·03
10 days	4	92·25 ± 1·97**	6·00 ± 2·27–**	1·00 ± 0·22–**	0·75 ± 0·48
14 days	4	93·75 ± 0·24**	4·25 ± 0·48–**	0·25 ± 0.24–**	1·75 ± 0·63
High ligation					
6 days	3	67·33 ± 19·33**	28·00 ± 25·50	2·67 ± 2·19–*	2·00 ± 0·58
10 days	4	96·75 ± 1·60**	1·75 ± 1·44–**	0·00–**	1·50 ± 1·50
14 days	4	92·25 ± 2·53**	1·50 ± 0·96–**	0·25 ± 0·24–**	6·00 ± 2·71
Controls (Cauda epididymidis)	8	94·00 ± 2·25	4·63 ± 2·21	0·25 ± 0·17	1·12 ± 0·44

† See text for description of different types.
* Significant difference ($0·01 < P < 0·05$) between corresponding figure and that for controls (–* where difference is one of reduction).
** Highly significant difference ($P < 0·01$) (–** where difference is one of reduction).

not exhibit this change until 10 to 14 days of ligation had elapsed. These changes are similar in principle to those seen in the low ligation group, but were more pronounced, for after 6 days samples from the caput epididymidis exhibited wave motion, whilst after 14 days virtually all spermatozoa from level 4a were already immotile.

Again, there was an inverse relationship between motility and eosinophilia in samples from each level, with the exception of level 4a where the proportion of eosinophilic spermatozoa was not significantly reduced at any time (*see* Table 1).

The incidence of spermatozoa with coiled tails followed a similar pattern in the initial stages of ligation to that seen in the low ligation group, namely an increase at 6 days in the region just proximal to the ligature, and a similar increase at 10 days in the caput epididymidis. By contrast, however, a high

incidence of these spermatozoa occurred in the initial segment also at 10 days, and the proportion was increased at 14 days.

Migration of the protoplasmic droplet occurred earlier in this group, for, after 10 days, counts on samples from level 1 indicated a significant decrease in the proportion of spermatozoa with proximal droplets. This was accompanied by a slight increase in the occurrence of spermatozoa with distal droplets (*see* Table 2). Droplet abnormalities of a similar type to those already described were again evident but they also appeared earlier than in the low ligation group (*see* Table 3) and were to be found just proximal to the ligature as well as in the head.

With regard to changes in the acrosome, counts on samples from level 2 showed that after 6 days there was a preponderance of type 1 acrosomes in two animals, whilst in a third animal, the proportions did not differ significantly from the controls. However, after 10 and 14 days, a high incidence of type 1 was found in all the experimental animals (*see* Table 4).

DISCUSSION

This work provides further evidence that obstruction of normal flow in the epididymis of the rabbit results in a distinct pattern of changes in the contained spermatozoa. This is characterized by the development of an increased capacity for in-vitro motility, first by those spermatozoa immediately above the site of occlusion and later by those at higher levels of the epididymis. Observations of the motility, morphology and differential staining reactions, however, all indicate that this apparent 'awakening' of the spermatozoa is soon followed by their degeneration. At all events, the findings suggest that when spermatozoa are arrested during their passage through the epididymis, they undergo *in situ* certain changes that are usually associated with maturation. That some of the changes occurred more rapidly after high ligation than low ligation is probably due to the earlier arrest of sperm flow in the epididymis following high ligation. Thus samples of spermatozoa from the initial segment, for instance, would show a large proportion of immature spermatozoa for a longer period of time after low ligation, because replenishment from the testis would not be blocked as quickly.

The question still remains as to whether changes induced by ligation of the epididymis concern only the motility and staining reaction of the spermatozoa or whether they truly represent so-called maturation changes. The morphological features of the spermatozoa were of value in this connexion. Results show that under the highly artificial conditions of ligation of the epididymis, protoplasmic droplets can migrate to the end of the mid-piece whilst the spermatozoa are still within the initial segment. Moreover, abundant spermatozoa with contracted acrosomes can also be found at the level of the caput flexure following epididymal ligation, whereas in the intact animal a preponderance of spermatozoa with expanded acrosomes is normally found at this level.

The 'cytoplasmic cap' which has been described in the spermatozoa of several domestic species (Rao, 1958) is probably analogous to the type 2 acrosome observed in this work, but suggestions that it is a normal feature (Rao & Berry, 1949) or that it denotes functional immaturity (McKenzie, Miller &

Bauguess, 1938) have been challenged by Hancock (1952, 1956). He believed that the presence of the 'cap' reflected a post-mortem change in the acrosome. However, in the present work it has been possible, with the use of Papanicolaou's staining technique, to distinguish two kinds of 'swollen' acrosome, and it is considered that these represent immature and dead spermatozoa. Thus dead spermatozoa could be discounted when assessing the proportions of mature and immature forms.

The data from the counts of acrosome types suggest that observed increases in the motility of spermatozoa following ligation of the epididymis reflect the degree of maturation. But further evidence from this study indicates that this is not a precise relationship.

For example, a number of spermatozoa develop an abnormality of their protoplasmic droplet. A significant increase in the incidence of this type of spermatozoon after 14 days is probably due largely to fragmenting droplets and may be purely a sign of degeneration, but many swollen droplets occurred earlier in association with coiled tailed spermatozoa. The presence of these is obscured in the data because they were counted as coiled tails only.

Secondly, the high incidence of spermatozoa with coiled tails, which occurred in both the high ligation and low ligation groups, is not a normal feature of maturation. For the purpose of this investigation all forms of contorted tails or mid-pieces were classified as 'coiled tails'. Swanson & Boyd (1962) in a study of coiled tails in both ejaculated and epididymal spermatozoa of bulls, reported that they were associated with poor motility. By contrast, the present investigation has shown that epididymal spermatozoa trapped in a ligated tubule for only a short period, become very actively motile, yet smears of such spermatozoa show a fairly high proportion of coiled tails. It is possible that some of these coiled tails are produced *in vitro*, for close observation of fresh samples before smearing showed that the spermatozoa did not have coiled tails at that time, although they exhibited an unusual form of convulsive movement. By comparison, the high incidence of coiled tails in smears taken at later stages of the experiment are more likely to have been actually produced *in vivo*, since they could be clearly seen in fresh samples which showed very poor motility.

The development of abnormal motility after epididymal ligation is of interest in connexion with the observations of Blandau & Rumery (1961) that, in the rat, spermatozoa from the head of the epididymis normally exhibit a form of movement which differs from that of mature spermatozoa. They suggest that this may have a bearing on the relative inability of immature sperms to fertilize, and it would certainly seem valuable to carry out fertility tests in the rabbit after epididymal ligation.

The time of onset of changes in the spermatozoa is also of interest, for contraction of the acrosome was seen to have taken place in many spermatozoa as early as 3 days following epididymal obstruction. Normally the rate of transit of spermatozoa through the epididymis of the rabbit is between 5 and 12 days (Edwards, 1940; Koefoed-Johnsen, 1961; Amann & Koefoed-Johnsen, 1963), but this concerns their passage from the testis to the ductus deferens, and most spermatozoa show signs of maturation by the time they enter the tail of the epididymis. It could well be, therefore, that rabbit sperms only require about

3 days to attain maturity and that ligation of the epididymis does not significantly affect this. Degeneration of the spermatozoa could also be interpreted as a mere extension of the maturation process.

In previous work there has been some doubt about the significance of eosinophilia in epididymal spermatozoa. Amann & Almquist (1962) believed it to be due to a change in the permeability of the cells, and, whilst this is undoubtedly so, the origin of such a change is more obscure. A high degree of eosinophilia is invariably associated with poor overall motility in fresh samples and many decapitated spermatozoa in smears, and, following ligation of the epididymis, it is strongly suggestive of degeneration and possible ageing of the spermatozoa. This may not, of course, apply to eosinophilic spermatozoa from intact epididymides.

The present results in the rabbit thus appear to be in accordance with the findings of Young (1931) in the guinea-pig, that spermatozoa can mature *in situ* when their normal flow is arrested by ligation of the epididymis, because increases in motility were accompanied by morphological changes that are normally associated with maturation.

Nevertheless, accumulation of fluid as well as of spermatozoa results from ligation of the epididymis, so that the environment of spermatozoa at any one level of the duct must differ from that in the normal animal. It is suggested, therefore, that the altered environment could be largely responsible for abnormal features in motility and morphology and that these are superimposed upon an otherwise normal process of maturation.

ACKNOWLEDGMENTS

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to say whether, following artificial cryptorchidism, retention of the droplet in live spermatozoa is a primary manifestation of intrinsic degenerative change in the spermatozoa, or whether it is a more complex response to a change in milieu within the epididymis.

It is interesting that fertility, in terms of foetal survival, appeared to be significantly improved after 1 day of artificial cryptorchidism. Salisbury & Flerchinger (1967) noticed a similar transient increase in the fertility of bull semen stored *in vitro*, and postulated that spermatozoa with 'aberrant chromatin' were the first to lose their fertilizing competence under these conditions. Even assuming that the reduced embryonic losses noted in the present work were due to a particular loss of fertilizing ability on the part of some spermatozoa, it is difficult to see how the genetic content of a spermatozoon could in itself influence the ability of the cell to withstand adverse environmental conditions, such as those imposed by artificial cryptorchidism. A possible explanation is that the oldest spermatozoa are the first to lose fertilizing competence, having probably suffered the most cytoplasmic and nuclear damage due to wear and tear.

It is known that rabbit spermatozoa take 9 to 12 days to pass through the epididymis (Koefoed-Johnsen, 1961) and there is evidence that considerable mixing occurs during this transit (Orgebin-Crist, 1965). It is probable, therefore, that the sperm population within the cauda epididymidis consists of cells with widely differing chronological ages. Observations in the bull (De Groot, 1961) and in the guinea-pig (Young & Simeone, 1930; Simeone & Young, 1931) have also indicated that 'ageing' of spermatozoa may become apparent within the epididymis before ejaculation. Artificial cryptorchidism did not appear to reduce the ability of spermatozoa to induce normal embryogenesis. This fact contrasts interestingly with the results of 'ageing' spermatozoa in the cauda epididymidis at scrotal temperatures. When migration of 'young' forms is prevented by ligation of the corpus epididymidis, the fertility of spermatozoa stored in the cauda is not lost for 28 to 49 days (Hammond & Asdell, 1926; Igboeli, 1968; Paŭfler & Foote, 1968; Tesh, 1968; Tesh & Glover, 1969), and furthermore, adverse effects on the viability of embryos are apparent before any decrease in the fertilizing ability of the spermatozoa. Similar patterns of functional change are known to accompany the storage of spermatozoa *in vitro* and irradiation of semen (Salisbury, 1965; Lanman, 1968). These indicate that sperm chromatin is not stable for an indefinite period but is susceptible to a slow degenerative change. Such change may show itself by death of implanted embryos. However, in artificial cryptorchidism, it is possible that the fertilizing ability of spermatozoa is so rapidly lost that any latent or actual genetic damage that might occur is masked. In this connection, it is noteworthy that when spermatozoa are 'aged' fairly quickly in the uterus of the rabbit, it appears to be the fertilizing capacity that is first affected (Hammond & Asdell, 1926; Tesh, 1968, 1969). It is tempting to consider that abdominal temperature might be the detrimental cause in both conditions but so many differences exist between the environments in the male and female tracts that, at this stage, the idea can only be conjectural. It is more likely that fertilization failure in both situations results from a stereotyped response of spermatozoa to fairly severe adverse environments

and does not necessarily reflect the action of a common factor. The present results suggest that one early effect of artificial cryptorchidism may be the inhibition of fertilizing capacity in chronologically 'old' spermatozoa. This would increase the probability of ova being fertilized by genetically sound gametes and, thus, initially improve fertility. However, even if this be true, there will be a rapid loss of fertility as the number of competent spermatozoa drops below the threshold needed for sperm–ovum contact.

At all events, there is little doubt that early loss of fertility following artificial cryptorchidism is due to a failure of sperm penetration rather than sperm transport. This contrasts with the suggestion made by Asdell & Salisbury (1941) and will be discussed in more detail elsewhere.

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Epididymal spermatozoa.

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Some aspects of the postnatal development of the epididymis in the rabbit.

By T. D. GLOVER and P. GADDUM. *University of Liverpool*

Histological examination of the epididymis during its postnatal development has been undertaken as part of a more extensive study of prepubertal changes in the male reproductive organs of the rabbit. It is known that distinct histological and cytochemical differences exist between various regions of the epididymis in the adult rabbit (Nicander, *Acta Morph. neerl. scand.* **1**, 1957), and the present investigation is concerned with the development of these regional differences during early post-natal life.

Male rabbits of mixed strain were killed in groups of two to three at fortnightly intervals from birth to 34 weeks. The testes and epididymides were weighed and fixed in Bouin's solution and sections of the testes and the various regions of the epididymides were stained with haematoxylin using either eosin or chromotrope 2R as a counterstain. The height of the epithelium and the diameter of the lumen in specific regions of the epididymis were measured by means of a simple eyepiece micrometer. These regions included the initial segment, the head of the epididymis, the proximal and distal parts of the body of the epididymis, and three regions of the tail.

It was found that the process of growth and differentiation of the tubules was relatively slow in the first 8 weeks after birth, but at 8–10 weeks, a phase of great activity began. This resulted in the appearance of the definitive regions of the adult epididymis at 16–18 weeks. It was apparent, however, that the epithelium in the head of the epididymis reached its adult height of about 30μ at 12–16 weeks, whereas that in the body did not appear to complete its growth until 20–22 weeks. This supports the findings of Reid (*Aust. J. Zool.* **7**, 1959) in the rat, that differentiation in the proximal regions of the epididymis is completed before that in the more distal zones.

Spermatozoa first appeared in the testes at 14 weeks, but did not fill the tubules of the tail until after 20 weeks. The earliest of these spermatozoa, which were preceded by a number of exfoliated testicular cells were not fully motile *in vitro*, and showed certain abnormal characteristics. This suggests that, despite the presence of spermatozoa in the epididymis, normal reproductive function had not at this time become fully developed.

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PROCEEDINGS OF THE SOCIETY FOR THE STUDY OF FERTILITY

Abstract of paper presented at Society for the Study of Fertility at the Annual Conference, 1966

The influence of ageing of rabbit spermatozoa on fertilization and prenatal development. J. M. TESH and T. D. GLOVER. *Faculty of Veterinary Science, Liverpool.*

Hammond & Asdell (1926) compared the vitality of spermatozoa in the male and female tracts of the rabbit. Their work demonstrated a direct relationship between the age of spermatozoa and loss of fertility, but provided no information as to whether the effect of ageing was concerned primarily with fertilizing capacity or resulted in post-fertilization failure.

The present work describes observations that have been made on the fertility of does following insemination with spermatozoa that have aged (a) in the male tract following bilateral ligation of the distal part of the body of the epididymis and (b) in the female tract by prolonging the normal interval between insemination and ovulation. In the second experiment, normal spermatozoa were inseminated at varying intervals before the induction of ovulation by HCG.

The fertilizing capacity of spermatozoa that had resided in the epididymis for periods up to 2 weeks was apparently normal, but was greatly reduced after 4 weeks and completely destroyed after 7 weeks. However, pre-implantation loss and post-implantation death were more striking effects and occurred before a significant failure in fertilization could be detected. A number of embryological abnormalities were also observed and will be discussed.

The spermatozoa of normal males retained their full fertilizing capacity in the female tract for a period of only about 15 hr before HCG injections (i.e. about 25 hr before ovulation), and embryonic development was normal. When the period was extended to 21 hr, however, fertilization was much reduced and an increasing amount of post-implantation mortality occurred. After 22 hr, fertilizing capacity was completely lost.

These results confirm the findings of Hammond & Asdell (1926) that rabbit spermatozoa survive for a longer period of time in the epididymis than in the female tract. It now seems that the first effect of spermatozoa aged in the male tract is to increase pre-implantation loss and post-implantation mortality, and later, when still older spermatozoa are used, there is a primary failure in fertilization. By contrast, ageing of spermatozoa in the female tract appears to exert its effect mainly on fertilization, and does not seem so seriously to affect post fertilization losses.

This work was aided by a grant from the Population Council Inc. (U.S.A.).

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J. Reprod. Fert. (1969) 20, 287–297

AGEING OF RABBIT SPERMATOZOA IN THE MALE TRACT AND ITS EFFECT ON FERTILITY

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(*Received 23rd September* 1968)

Summary. The influence on fertility of the ageing of rabbit spermatozoa in the male tract has been investigated. To age the spermatozoa, the corpus epididymidis was ligated bilaterally so that the inflow of younger spermatozoa into the tail of the epididymis from higher levels of the duct was prevented. Ejaculated semen samples collected from the rabbits at different times after the operation were used for insemination. It was found that the fertilizing capacity of the spermatozoa was lost after 49 days. Losses before implantation increased progressively until 28-day-old spermatozoa were used, but post-implantation losses reached a peak with 35-day-old spermatozoa. After implantation, losses appeared to occur at progressively earlier stages of development as the age of the spermatozoa increased. In the strain of rabbits used, several foetuses were seen to lack gall bladders and to have abnormal skull sutures following the insemination of does with aged spermatozoa.

Throughout the experiment, the motility, morphology and reaction of the spermatozoa to staining with aqueous nigrosin–eosin was examined. The significance and possible causes of adverse changes in ageing spermatozoa are discussed.

INTRODUCTION

The tail of the epididymis is a storage area for mature spermatozoa (Von Lanz, 1924, 1926; Polovtzeva, 1938; Chang, 1945; Ortavant, 1952; Chang & Schaeffer, 1957), but although the conditions are favourable to their survival, spermatozoa are not preserved indefinitely in a viable state; gradually they age and so lose their vitality (Hammond & Asdell, 1926; Young, 1929, 1931). There are appreciable differences between species in the length of time spermatozoa can survive in the male tract. In the mouse, fertility is not affected until spermatozoa are 10 to 14 days old (Snell, 1933), whilst in the rat they must be 20 days old (White, 1933) and in the guinea-pig 20 to 35 days old (Young, 1929). In the rabbit, spermatozoa must be over 40 days old before complete sterility results (Hammond & Asdell, 1926).

Unless the older spermatozoa are eliminated from the male tract quickly and efficiently, their incidence in ejaculated semen is likely to vary according to the

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frequency of sexual activity. This means that after long periods of sexual rest, old or ageing spermatozoa might well preponderate in the first few ejaculates. The evidence is that, if this were to happen, infertility might result, for the work of Young (1929, 1931) in the guinea-pig suggests that, even if fertilization were achieved, aged spermatozoa might cause embryonic mortality. It appears that in guinea-pigs, 20- to 25-day-old spermatozoa may give rise to an increase of almost 20% in the incidence of non-viable embryos.

These effects have not been clearly demonstrated in the rabbit, for Hammond & Asdell (1926) used the number of pregnancies and the size of litters as criteria of fertility. It was hoped that, by undertaking a more detailed investigation of ageing in rabbit spermatozoa, its influence on prenatal development as well as on fertilizing capacity could be determined. The rabbit has an additional advantage as an experimental animal in that ejaculated semen is readily obtained and appraised, and changes in semen quality can be judged in relation to ensuing levels of fertility.

MATERIALS AND METHODS

Fifteen adult bucks and ninety-seven does were used. They were of the New Zealand White strain from a closed colony, and varied in weight from 3·75 to 4·50 kg. They were maintained under the same conditions of environment and diet (Diet SG1, Oxoid Ltd, supplemented with hay). Permanent access to drinking water was provided for each animal.

Procedures with male animals

Bilateral ligation of the epididymis was carried out in each of the bucks used in the experiment. They were anaesthetized with nitrous oxide and halothane (Fluothane, ICI Pharmaceuticals Ltd) which was administered by a face mask using the open circuit method. Nitrous oxide and oxygen in a ratio of 4:1 and at a flow rate of 2·5 litres/min were used to induce anaesthesia, and a 5:2 ratio of nitrous oxide and oxygen containing 5 to 6% halothane at a flow rate of 700 ml/min, was used to maintain it.

An incision of approximately 1 cm in length was made in the scrotal skin and tunica vaginalis on the lateral surface of the scrotal sac. The body of the epididymis and part of the tail of the epididymis were immediately visible. Two ligatures were placed around the distal portion of the body of the epididymis approximately 0·5 cm apart and the intervening segment was removed. Care was taken to avoid damage to associated blood vessels (Glover, 1960). There was no handling of the epididymis, except for the small portion that was ultimately removed as part of the operation. The scrotal approach is much less traumatic and therefore preferable to the abdominal approach which involves elevating the testes and subsequently returning them to the scrotum (Glover, unpublished observations).

The bucks used in this experiment were selected for the high quality of their semen. Collections of semen were made using an artificial vagina (Walton, 1958). Control ejaculates were collected from each animal 2 days before operation, and post-operatively they were taken at 7, 14 and 21 days from seven

of the bucks and at 28, 35, 42 and 49 days from the remaining eight animals. In this way spermatozoa were obtained that had been 'aged' in the cauda epididymidis and ductus deferens from 0 to 49 days.

Individual samples taken at any one of the post-operative periods were examined for motility by simple microscopical observation. They were then pooled, diluted to 10 ml with physiological saline and the total concentration of spermatozoa was determined. Motility was again checked after dilution, and smears stained with aqueous nigrosin–eosin (Glover, 1960) were prepared. From these smears, certain morphological features of the spermatozoa and the incidence of eosinophilic forms were examined. Morphologically abnormal spermatozoa included decapitate forms, spermatozoa with coiled tails, distorted mid-pieces and abnormal features of the acrosomes. Although 'temperature-shock' (Hancock, 1951) does not appear to be a problem with rabbit spermatozoa (Wales & White, 1959), there was the possibility that ageing might render the spermatozoa more susceptible to sharp changes in temperature. For this reason, the stain and equipment were warmed to about 30° C before use.

Procedures with female animals

The does in this experiment were caged singly from 12 weeks of age and on reaching 20 weeks of age they were injected intravenously with 25 i.u. luteinizing hormone (Pregnyl, Organon Laboratories) to induce a state of pseudopregnancy. This terminated with the onset of oestrus 19 to 21 days later (Adams, 1960).

Age-changes in the spermatozoa were then tested by insemination of these does. At each of the eight stages in the 0- to 49-day period of sperm ageing, twelve to fourteen does were each inseminated with 0·5 ml of pooled semen and immediately given a further injection of LH. On every occasion, half the does in a group were selected at random and 48 to 60 hr afterwards they were killed with a rapid injection of sodium pentobarbitone (Nembutal, Abbott Laboratories). Eggs were flushed from these animals and examined for cleavage which was taken as evidence of fertilization (Venge, 1950; Adams, 1956; Tesh, 1966). The eggs were first examined fresh and then, after fixation with acetic alcohol, they were stained with aceto-carmine (Tesh, 1966). The fertilization rate of the spermatozoa (i.e. the percentage of eggs fertilized) at each of the eight periods of ageing was thus determined and was expressed as a mean figure for each period. The does concerned were referred to as Group 1. The remaining does, Group 2, were used to assess effects on post-fertilization development. For this, the does were examined by laparotomy on Day 10 following the ovulatory injection of LH, in order to determine the number of implantation sites. At this stage, implantations were seen as discrete swellings 12 to 21 mm in diameter (Adams, 1960). When the implantation sites had been counted, the abdomen was closed using sterile braided surgical silk, size 00. This procedure took between 12 and 20 min to complete so that possible adverse effects on foetuses were reduced to a minimum. Nitrous oxide and halothane anaesthesia was again used since it is known that barbiturates might, in some circumstances, affect foetuses (Tesh, unpublished observations; Champakamalina & Appaswamy Rao, 1967).

The pregnancy was allowed to continue until Day 29 when the does were killed and detailed examination of the contents of each uterus was made. The number, weight and sex of live foetuses were recorded together with the weights of both normal and atrophic placentae. The number, weight and size of dead foetuses were noted and, if present, their position in the uterus was recorded. Live foetuses were killed with an overdose of pentobarbitone sodium and a detailed macroscopical examination was then performed. Foetuses showing abnormal features, particularly skeletal deformities, were fixed and stained with a modification of Dawson's staining technique (Tesh & Worsley, unpublished observations). The ovaries of each doe were removed and the corpora lutea were counted. From all these data, post-fertilization failure was estimated.

By multiplying fertilization rate, as assessed from animals in Group 1, by the number of corpora lutea counted in Group 2, an estimate was made of fertilization rate in Group 2 does. The number of implantation sites seen on Day 10 and the number of live foetuses on Day 29 were each divided by this estimate in order to calculate both pre-implantation failure and post-implantation loss. The calculations were based as follows:

$$\% \text{ Fertilization} = \frac{\text{No. of eggs fertilized}}{\text{Total no. of eggs obtained}} \times 100$$

$$\% \text{ Implantations} = \frac{\text{No. of implantations}}{\text{Estimated no. of eggs fertilized}} \times 100$$

$$\% \text{ Live foetuses} = \frac{\text{No. of live foetuses at Day 29}}{\text{Estimated no. of eggs fertilized}} \times 100$$

From these calculations, failure in fertility could be defined as:

Fertilization failure = 100 minus% fertilization
Pre-implantation failure = Estimated no. of eggs fertilized minus No. of implantation sites
Post-implantation loss = No. of implantation sites minus No. of live foetuses at Day 29

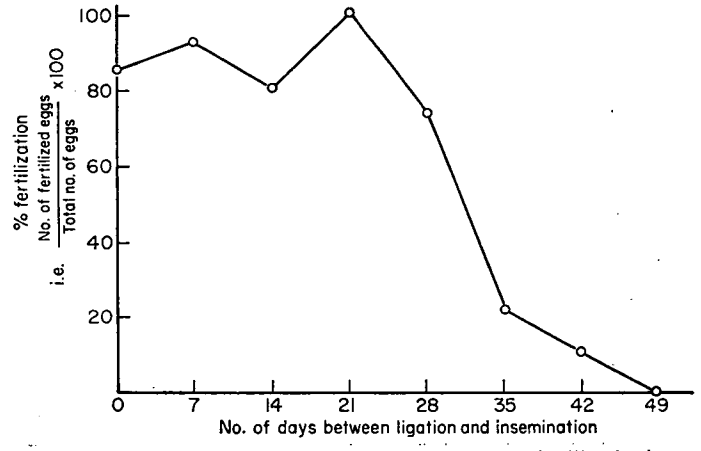
Further refinement, according to the criteria of Adams (1962), involved a more precise definition of the stage of post-implantation failure. Damage that occurred shortly after implantation resulting in complete resorption by Day 29 was called 'early' loss, and that occurring between this time and about Day 17 of gestation was considered as 'middle' loss. This was recognized on Day 29 by the presence of atrophic placentae. 'Late' loss represented damage occurring later than Day 17 and this usually gave rise to dead foetuses.

RESULTS

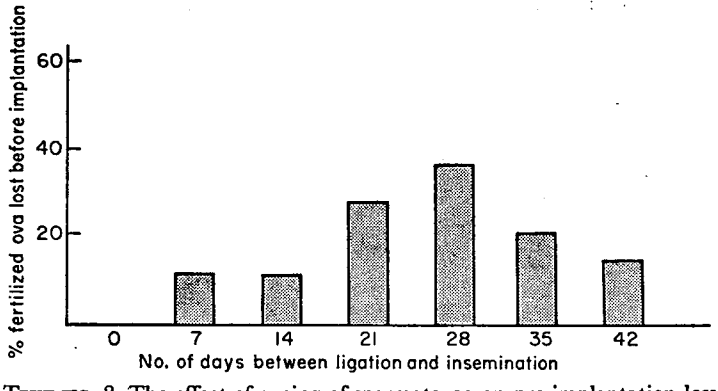
Effects on fertilization

Text-figure 1 shows data obtained from animals in Group 1 and illustrates the influence of ageing in spermatozoa on the fertilization rate. This was found to be unaffected until 28-day-old spermatozoa were used but between these and 35-day-old spermatozoa, the fertilization rate decreased dramatically from

73·8% to 21·9%. Thereafter, the decrease continued at a slower rate, until 49-day-old spermatozoa were shown to have completely lost their capacity to fertilize (Text-fig. 1). Even so, 49-day-old spermatozoa were seen in the zona pellucida and perivitelline space of uncleaved eggs, showing that some of them were at least capable of reaching the site of fertilization and penetrating the envelopes of the egg.



TEXT-FIG. 1. The effect of ageing of spermatozoa on fertilization rate.



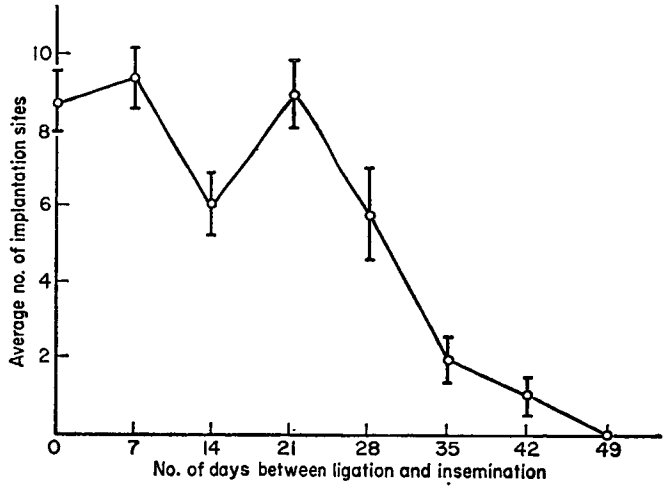
TEXT-FIG. 2. The effect of ageing of spermatozoa on pre-implantation loss.

Effects on pre-implantation development

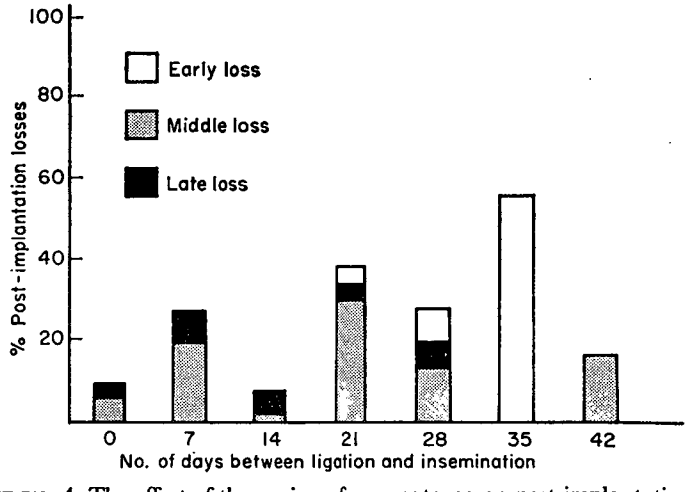
The extent of disintegration or death of conceptuses before implantation is shown in Text-fig. 2. There was no pre-implantation loss in control animals but in those inseminated with spermatozoa from operated bucks, it increased progressively with the increasing age of the spermatozoa. A maximum of 38·3% was reached with 28-day-old spermatozoa but 42-day-old spermatozoa caused only 16·4% loss. There appeared to be no effect on the actual number of implantation sites, until the spermatozoa were 28 days old. Between 21 and 28 days of ageing, the mean number of implantation sites decreased from 9·0 to 5·8. This rapid decline in implantation rate continued until fertility was completely lost after 49 days of ageing (Text-fig. 3).

Effects on post-implantation development

Post-implantation loss was calculated as the percentage of implantations lost before autopsy on Day 29 of gestation, and the results are shown in Text-fig. 4. It seems that the overall post-implantation loss increases when progressively older spermatozoa are used, but when spermatozoa of more than 35 days of age are used this loss appears to decline.



TEXT-FIG. 3. The effect of the ageing of spermatozoa on the implantation of blastocysts.

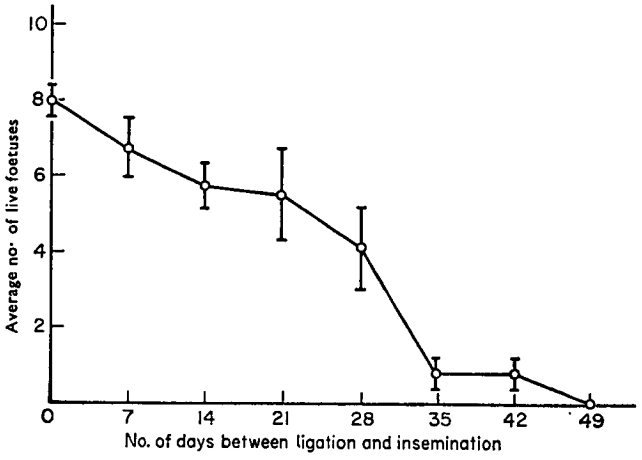


TEXT-FIG. 4. The effect of the ageing of spermatozoa on post-implantation loss.

When the distribution of post-implantation losses was examined, it was found that, when the spermatozoa had been aged for up to 14 days, the loss occurred at the 'middle' and 'late' stages of development only. When the time of ageing was extended beyond this, there was some 'early' loss and this gradually increased to a peak of 57·2% with 35-day-old spermatozoa. Increased 'middle'

losses were associated with this 'early' loss, but when the spermatozoa had been aged for 35 days or longer there was no loss at the 'late' stage of development.

The mean number of foetuses per female showed a progressive decrease as age of the spermatozoa increased, and became significantly different from the controls when the spermatozoa had been aged for 28 days ($P<0{\cdot}05$). Between 28 and 35 days of ageing, the decrease became more rapid and the mean number of foetuses per female declined abruptly from 4·1 to 0·8. The number was maintained at this level until fertility was entirely lost at 49 days (Text-fig. 5).



TEXT-FIG. 5. The effect of the ageing of spermatozoa on the number of live foetuses present in the uterus on Day 29 of pregnancy.

Although there was no difference in mean foetal weights caused by ageing spermatozoa, a highly significant negative linear regression was established between mean foetal weight and the number of foetuses in a litter.

No significant differences in the ratio of male to female foetuses occurred at any stage but the incidence of foetuses without a gall-bladder, and particularly those with abnormal parietal skull sutures, did seem to be related to increasing age of the spermatozoa.

Characteristics of spermatozoa

Examination of the spermatozoa before insemination revealed that up to 28 days following ligation, there was no change in motility or in the incidence of eosinophilic spermatozoa but that between 28 days and 49 days, there was a decline in motility and an increased proportion of eosinophilic spermatozoa. This, together with other semen characteristics, is shown in Table 1.

The percentage of unstained spermatozoa with coiled tails was relatively constant in semen samples until the 28th day of ligation, when it suddenly increased from 2·5% to 23·0%. This was reversed at Day 35 when a marked increase in the incidence of stained and decapitate spermatozoa took place. It may be taken, therefore, that after this time ejaculates contained a preponderance of dead spermatozoa.

TABLE 1

CHARACTERISTICS OF SPERMATOZOA AGED IN MALE TRACT

No. days aged	Motility	Unstained				Stained			
		Normal	Coiled tails	Decapitates	Other abnormalities	Normal	Coiled tails	Decapitates	Other abnormalities
0	++++	81·0	1·0	1·0	2·0	12·0	1·0	2·0	0·0
7	++++	78·5	0·5	1·0	0·0	16·0	0·5	3·5	0·0
14	++++	82·0	1·7	0·0	3·7	8·6	0·0	3·0	1·0
21	++++	79·5	2·5	0·0	1·0	9·0	4·0	4·0	0·0
28	++++	52·5	23·0	0·0	0·5	7·5	3·5	11·5	1·5
35	++++	32·0	3·8	0·5	0·5	31·1	2·8	29·3	0·0
42	+++	47·8	2·8	0·5	0·0	24·9	5·0	20·0	0·0
49	++	11·0	6·6	0·4	0·0	30·8	15·6	35·6	0·0

DISCUSSION

This work confirms earlier evidence in laboratory animals (Hammond & Asdell, 1926; Young, 1929, 1931; White, 1933) that infertility may be caused by spermatozoa that have been artificially aged in the male tract for more than about 3 weeks but does not support the view of Hammond & Asdell (1926) that, in the rabbit, ageing in spermatozoa causes no increase in embryonic death. These authors, however, used successful pregnancies as their indicator of fertility and their comments on embryonic loss were based on two does only. Young (1931) found that, in the guinea-pig, an increased incidence of foetal resorptions and abortion was associated with the use of aged spermatozoa, but that this was not apparent when the ability to produce live young was taken as the only criterion of fertility. It does seem, therefore, that some of the effects of ageing in spermatozoa may not become apparent until after implantation of the blastocyst, but, in the rabbit at least, there has hitherto been no clear information on this or on how the ageing of spermatozoa might affect fertilization or pre-implantation development.

The present experiment has provided information on these points, though the precise cause of events is obscure. No firm conclusion can be drawn from the pre-implantation losses because of variability and possible error incurred by the method of calculation. In the case of post-implantation losses, the age of the spermatozoa seemed to be inversely related to the age of the embryos that were lost. It must be assumed, therefore, that some of the earliest changes in the ageing of spermatozoa concern the genome. These changes are distinct from, if additional to, presumed deterioration of the acrosome which will affect sperm penetration and, if widespread, will ultimately cause total failure of fertilization. Ageing of bull spermatozoa *in vitro* has been shown to bring about a change in the reaction of the spermatozoa to Feulgen staining and to influence prenatal losses (Salisbury, Birge, de la Torre & Lodge, 1961; Salisbury, 1967) and there is evidence also that, in the rabbit, ageing *in vivo* of epididymal spermatozoa influences the DNA content of the spermatozoa (Bouters, Esnault, Salisbury & Ortavant, 1967). There is, thus, considerable support for the notion that the ageing of spermatozoa affects their DNA content.

The present findings on the influence of age on sperm motility, morphology

and permeability to eosin, are largely in agreement with those of Gaddum (1965), except that, in the present work, signs of degeneration in the spermatozoa were observed sooner. Ejaculated spermatozoa were examined here, however, rather than epididymal spermatozoa. It appears that spermatozoa rendered effete by being aged in the tail of the epididymis, die and disintegrate in the ductus deferens or during ejaculation. There is evidence in the guinea-pig that the ductus deferens may not be as favourable to sperm survival as the tail of the epididymis (Simeone & Young, 1931).

If old spermatozoa do disintegrate in the ductus deferens, poor semen quality could be an early outcome of prolonged sexual rest unless aged spermatozoa could be removed very efficiently. On the other hand, if spermatozoa degenerate most rapidly in the tail of the epididymis, the possible effects of continence on the quality of ejaculates would be less evident since there would be more time for mixing of spermatozoa in the tract (Orgebin-Crist, 1965) and for their absorption or removal.

Amann & Almquist (1962) held the view that bovine spermatozoa are eventually absorbed from the tail of the epididymis, but Gaddum & Glover (unpublished findings) have not been able to confirm this in rabbits. Roussel, Stallcup & Austin (1967) showed that decapitate spermatozoa may be phagocytosed in the cauda epididymidis of bulls, rabbits and monkeys and this undoubtedly supports the findings of Amann & Almquist (1962). By contrast, Lino, Braden & Turnbull (1967) believed that in rams, spermatozoa are removed in the urine during sexual abstinence at a rate which corresponds to sperm production. Species and individual differences may explain, in part at least, the conflicting results obtained by different workers.

Walton (1933) thought that the first few ejaculates after sexual rest might contain an excess of poor quality spermatozoa, but this was not substantiated by Gunn (1936) in rams. Nevertheless, this sort of effect has been reported in the ram by Phillips & McKenzie (1934) and McKenzie & Berliner (1937), and also in the bull by Webster (1932) and Herman & Swanson (1941), in the stallion by Polowzow (1927) and in the boar by Anderson (1945). While adverse effects caused by old spermatozoa are also demonstrated by the present work, it cannot be assumed that prolonged sexual rest will inevitably lead to subfertility. It is by no means clear how far the present work reflects changes in spermatozoa that occur in the normal intact animal. In such an animal, not only younger spermatozoa but also tubular fluid can freely flow into the tail of the epididymis, and when normal mixing of spermatozoa is permitted, their rate of ageing might be effectively reduced. Even so, an egg might at any time be fertilized by an abnormally old spermatozoon, so that it is valuable to know what possible effects might result.

A significant finding in this investigation was that fertility was impaired even though the number of actively motile and ostensibly normal spermatozoa was always in excess of the 0·5 to 1 million needed for maximum fertility (Wales, Martin & O'Shea, 1965; Hupp, personal communication). This was well shown when 49-day-old spermatozoa were tested. No fertilized ova were recovered from the does in spite of the presence of more than 3 million apparently normal spermatozoa in the inseminate.

Thus, although peak pre-implantation loss coincided with an increase in the incidence of spermatozoa with coiled tails in ejaculates, and the highest level of post-implantation loss was associated with an increase in the proportion of eosinophilic and decapitate spermatozoa, these abnormalities were not in themselves solely responsible for the infertility caused. It is suggested that they also indicated some deficiency in the apparently normal spermatozoa in the same ejaculates. In this way, it is believed, the abnormal spermatozoa revealed a more generalized deterioration of semen quality.

ACKNOWLEDGMENTS

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Some Aspects of Function in the Epididymis

EXPERIMENTAL OCCLUSION OF THE EPIDIDYMIS IN THE RABBIT

T. D. GLOVER (England)

It is well known that in most mammals spermatozoa undergo a process of maturation during their sojourn in the epididymis. This process is manifest in a number of ways, the most striking of which, perhaps, is the acquisition of motility in vitro and the development of a capacity to fertilize an ovum. Normally, therefore, only those spermatozoa in the tail of the epididymis are able to fertilize. In 1931, however, Young[10] showed that when the epididymis of the guinea pig is ligated and normal flow from the testis is thus arrested, the spermatozoa are seen to be fertile and vigorously motile when taken from any level of the duct or even from the testes. It appears then that spermatozoa can mature even in the head of the epididymis if they are not allowed to move along the duct, and this has been partially confirmed in the rabbit.[1, 5] Thus, maturation appears to be primarily a temporal phenomenon and specific activity of the duct epithelium at any one level of the epididymis would not seem to be vital to the process.

The epithelium of the epididymis is nevertheless profoundly affected by ligation of the duct, for it soon becomes distinctly flattened.[1, 6] There is some evidence that this is merely a pressure effect, but, even so, it might affect function in the duct. In the present work on rabbits, therefore, it was decided to occlude the epididymis for relatively long periods to assess the resulting function of the testis and epididymis.

MATERIALS AND METHODS

Ligation of the epididymis has been performed on more than 70 rabbits including those on which the present observations were made. The operation, carried out under sodium pentobarbitone (Nembutal, Abbotts' Laboratories) anaesthesia, consists simply of passing a ligature round the ductus epididymidis in the region of the lower corpus or caput epididymis. Each is referred to, respectively, as "low ligation" or "high ligation." Care is always taken to avoid undue damage to the blood vessels in the area, particularly the arteries, and this necessitates some appreciation of the distribution of epididymal arteries.[6] In the rabbit, it is important that the operation be done through the scrotum, for in this way neither the testis nor the epididymis need

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be handled. By contrast, an abdominal operation necessitates delivery of the testis and epididymis through the inguinal canals. While this can be done with great facility in the rabbit, return of the organs into the scrotum incurs trauma which is frequently sufficient to influence the usual response of the spermatozoa to duct ligation, and is therefore likely to give variable and unreliable results.

In the present work, 3 animals were killed at each time interval of two, four, eight, and 16 weeks after unilateral ligation of the epididymis. In each animal one epididymis was left intact to serve as a control. After death of the animal, spermatozoa were taken from the head of the epididymis and examined for motility. Morphologic examinations of the spermatozoa were made after staining with aqueous nigrosin eosin or Papanicolaou's stain.[5] Sections of these areas of the epididymis were taken for histologic examination, fixed in Bouin's solution, and stained with Mayer's hematoxylin and chromatrope 2 R. After removal of the associated epididymis, each testis was weighed and sections were prepared for histologic study in the same way as those of the epididymides.

More recently, thin sections have been taken from glutaraldehyde-fixed specimens of epididymis.

Results

Macroscopic changes

Ligation of the epididymis causes a characteristic distension of the duct above the level of ligation, as illustrated in Figure 1. This is due to the accumulation of spermatozoa and fluid. Within days, this distension extends into the seminiferous tubules themselves, some of which can be seen as white streaks on the surface of the testis. Distension in some of the central tubules of the testes is still apparent after two weeks of ligation of the duct.

Weight changes in the testes

Corresponding with the enlarged seminiferous tubules, the total weight of testes increases after two weeks of epididymal ligation, when compared with controls. After four weeks of ligation, however, testis weight decreases markedly and continues to be low over an eight-week period. After 16 weeks, testis weight shows distinct signs of returning to normal. These results are shown in Table I.

Histologic changes in the testis

The histologic changes in the testis resulting from ligation of the epididymis are shown in Figure 2.

Two Weeks (*Figure 2B*). After two weeks of epididymal ligation, there is obvious pressure in the seminiferous tubules of the testis which displaces the spermatozoa toward the periphery of the tubules and pushes them up against the germinal epithelium. All stages of spermatogenesis are present, although the over-all height of the germinal tissues is very much reduced. It would

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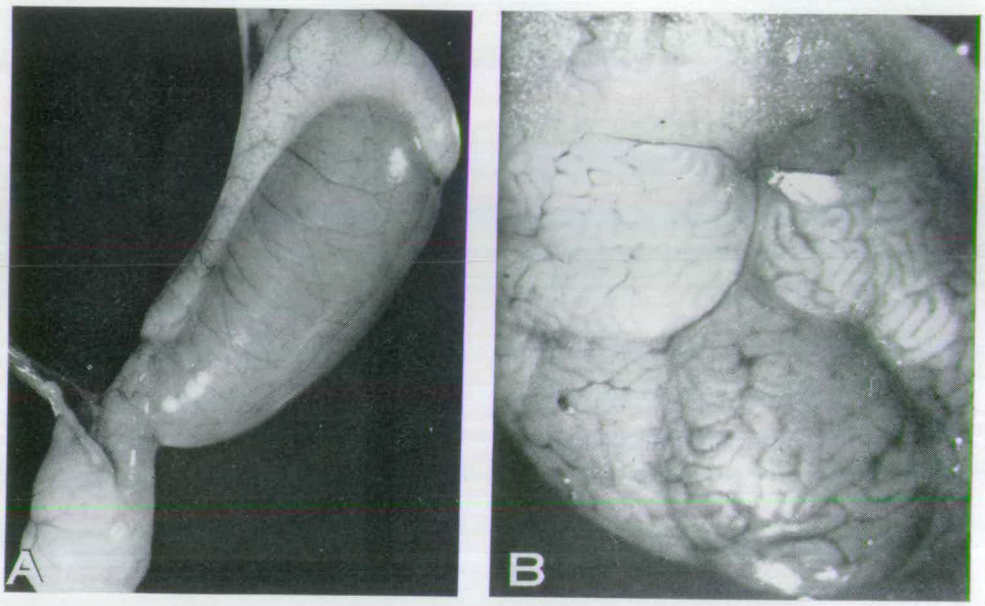

Figure 1. A. Appearance of epididymis two weeks after low ligation (see text). *B.* Appearance of head of epididymis (magnified) two weeks after low ligation.

seem that the lumen of the tubule is filled with fluid, although if this is so, the fluid does not stain with chromatrope 2 R. The degree of distension in different tubules varies considerably, but the over-all picture is characteristic.

Four Weeks (*Figure 2C*). At this time, definite signs of impaired spermatogenesis are evident. Distension of the tubules is not apparent; on the contrary, they are relatively collapsed, few if any spermatozoa are to be seen in the lumen, and profound changes in the germinal epithelium can be seen. Spermatocytes show large pyknotic nuclei, there is some coalescence of cells, and vacuolation is a prominent feature.

Eight Weeks. Although many tubules still show disturbed spermatogenesis at this time, there are signs of recovery in some of them. Germinal epithelium is higher in some tubules, and some spermatids and even spermatozoa are to be seen. The recovery must be only slight, however, since testis weight still appears to be reduced at this time.

Sixteen Weeks. (*Figure 2D*). At this time many tubules show active spermatogenesis and the height of the germinal epithelium is normal.

EVENTS IN THE EPIDIDYMIS

The acquisition of in vitro motility by the spermatozoa following epididymal ligation is relatively short-lived, and after three weeks they show virtually no movement when exposed to air. At this time and thereafter, a progressively increasing number of senescent and degenerated spermatozoa are to be found above the site of ligature, and the pattern of degeneration seems to be characteristic. Most of the spermatozoa are eosinophilic, several are decapitate, but

TABLE I

Changes in Testis Weight following Ligation of the Epididymis

Mean differences in weight between experimental and control testes; 3 animals per group.

Time after Ligation	*Weight Difference**	*Range*
wk.	*gm.*	*gm.*
2	+1.29	0.54–2.05
4	−1.20	0.94–1.46
8	−1.57	1.25–1.90
16	−0.87	0.61–1.13

* + = increase in testis weight; − = decrease in testis weight.

many show the tail to be coiled up behind the head. This feature has been shown previously,[7] but in the present work, electron micrographs have revealed that these tails are coiled up under the cell membrane of the spermatozoon, an effect reported previously following exposure of sperms to hypotonic media.[4] Moreover, electron micrographs indicate that after a month of epididymal ligation, the lumen of the duct contains many degenerated sperm tails (characterized by early loss of the axionema), and these appear to be removed by some form of phagocytosis which, it seems, may include absorption by cells of the epididymis.

Discussion

These results confirm earlier reports that occlusion of the epididymis allows the contained spermatozoa to mature at any level of the duct, but that the abnormal environment so produced causes a fairly rapid deterioration in the spermatozoa. Initial back pressure on the testis is evident, but present findings indicate that this too is only transitory. The observation that spermatogenesis is resumed, albeit only after 16 weeks, suggests that excess fluid and even spermatozoa must be removed either from the testes or epididymides or both.

Carr *et al.*[2] and Clegg and Macmillan[3] have demonstrated phagocytic activity in the testis of the rat, and attribute it to Sertoli cells. The present work now provides evidence of degenerated spermatozoa being removed partly by phagocytic bodies in the lumen[9] and possibly also by cells of the epididymis themselves. More work needs to be done on this, but initial electron micrographs suggest that incorporation of sperm tails into epididymal cells is possible, and it is tempting to speculate that sperm remnants may be removed by lysosomal activity in the epididymal cells.

The relevance of these experiments in rabbits to clinical problems in man lies in their relation to spontaneous epididymal obstruction. If the response in man to occlusion of the epididymis is similar to that in the rabbit, the present results mean that spermatozoa might initially mature above the site of an occlusion or stenosis of the epididymis. Most cases of epididymal obstruction in human patients will be seen after the duct has been occluded for months

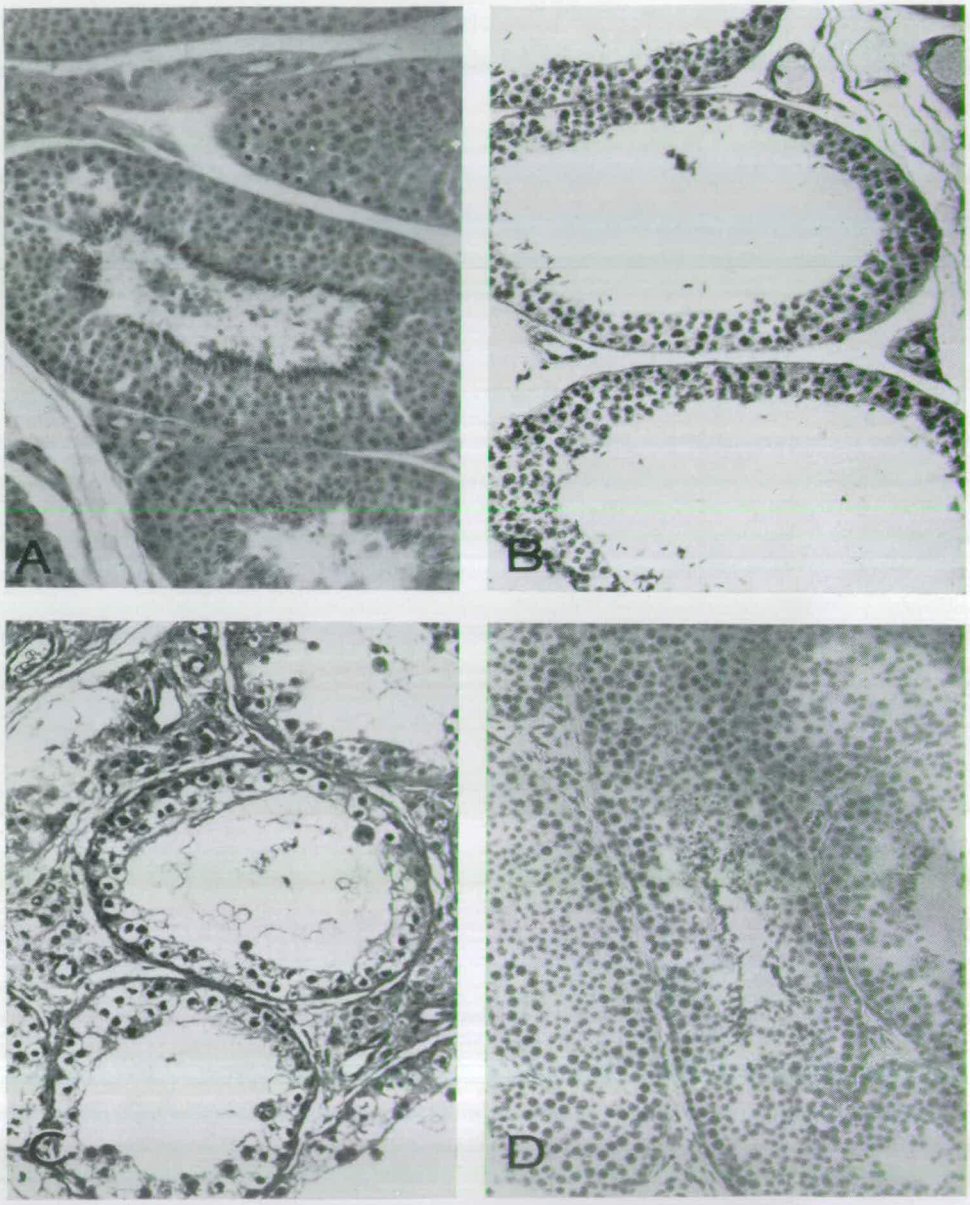

Figure 2. A. Section of control rat testis, showing normal spermatogenesis (250 ×). *B.* Section of testis following two weeks of epididymal ligation, showing distension of tubules (see text) (250 ×). *C.* Section of testis following four weeks of epididymal ligation, showing impaired spermatogenesis resulting from initial pressure (250 ×). *D.* Section of testis following 16 weeks of epididymal ligation, showing tubules with renewed spermatogenic activity (250 ×).

or even years, but it would seem from present results that active spermatogenesis might still be expected under these conditions, particularly if the obstruction is fairly low in the duct and close to the tail of the epididymis. If this is true, then the validity of vasoepididymostomy in the treatment of epididymal block is confirmed, and the possibility that mature spermatozoa might be ejaculated following the operation cannot be discounted.

Thanks are due to T. Williams for technical assistance.

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ALKALINE PHOSPHATASE IN THE CYTOPLASMIC DROPLET OF RABBIT SPERMATOZOA

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The presence of acid and alkaline phosphatase has been demonstrated biochemically in bull spermatozoa (Roussel & Stallcup, 1966), and lysosomal acid phosphatases have been shown to occur in the cytoplasmic droplet of bull and ram spermatozoa (Dott & Dingle, 1968). Both types of phosphatases have also been shown histochemically in the spermatozoa of bulls (Krause, 1969) and several other mammalian species (Wislocki, 1949; Bern, 1949). Allison & Hartree (1969) have referred to acid phosphatase being concentrated in the acrosome of vertebrate and invertebrate spermatozoa but there appears to be no comprehensive information about the localization of alkaline phosphatase in spermatozoa. The present paper reports on such a localization in rabbit epididymal spermatozoa.

During an investigation into the histochemistry of the rabbit epididymis, it was noticed that the cytoplasmic droplets of epididymal spermatozoa reacted positively when stained with Gomori's method, using sodium-β-glyceryl-phosphate as the substrate. The reaction depended upon the pH being strongly alkaline (preferably 9·6), and it was totally inhibited by the addition of cysteine (4 to 8 mM) to the substrate. The cytoplasmic droplets of testicular spermatozoa were also seen to react to staining with alkaline phosphatase (Plate 1a), but subjectively, proximal and migrating droplets did not appear to react as strongly as spermatozoa from the cauda epididymidis (Plate 1b), where, typically, the droplet has reached the end of the mid-piece and taken up an eccentric position on the flagellum.

Alkaline phosphatase not only occurs in spermatozoa from intact epididymides but also in those from a ligated epididymis. After 14 days of epididymal ligation, many spermatozoa in the ductuli efferentes possess cytoplasmic droplets that have already migrated to the end of the mid-piece, even though they are often distorted when conventionally stained (Gaddum & Glover, 1965). The present work has shown that these droplets also have considerable alkaline phosphatase activity.

It is of interest that in rabbit spermatozoa, alkaline phosphatase is concentrated exclusively in the cytoplasmic droplet, since it detracts from any categorical assertion that the droplet is entirely inert. Combine with this the membranous nature of cytoplasmic droplets, and the possibility of some local transport mechanism emerges (Morgan, Post & Park, 1960; Coleman & Finean, 1968).

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It is possible, of course, that the enzyme could simply be left over from the original spermatid cytoplasm but there is evidence that, in other areas of the male genital tract, alkaline phosphatase is associated with the transport of sugars across membranes (Bern, 1949; Mann, 1964), and the possibility of it being active in the cytoplasmic droplet should not be discounted. Since there is an abundance of alkaline phosphatase activity in the epididymal epithelium (Bern, 1949; Nicander, 1957a, b; Linnetz & Amman, 1968) and in the cytoplasmic droplets, it would be attractive to propose that the cytoplasmic droplets might in some way utilize the glycogen which is also present in the epithelium (Nicander, 1954) for the benefit of contained spermatozoa. All this must remain speculative until new evidence is available, but it would seem worth-while paying some attention to this rather curious occurrence of enzyme activity.

The present result may seem surprising in view of the report by Stambaugh & Buckley (1969) that they were unable to demonstrate phosphatases quantitatively in rabbit spermatozoa. It might be that the content in the droplet is too small to give a reading with the method used by these workers. Alternatively, differences in substrate might be involved for it is noteworthy that o-carboxyphenylphosphate used by these workers for both acid and alkaline phosphatase determinations differed from the substrate used in the present work and also from that employed by Dott & Dingle (1968).

Experiments are now being planned to examine the occurrence of alkaline phosphatase in degenerating epididymal spermatozoa and to test the effects of ejaculation frequency on the reaction. It might also be valuable to examine the distribution of alkaline phosphatase in ejaculated spermatozoa, provided a satisfactory technique for demonstrating it could be devised. It is important to know, too, whether this strict localization of alkaline phosphatase in cytoplasmic droplets is widespread among epididymal spermatozoa of mammals, or whether it is only characteristic of certain species.

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PLATE 1

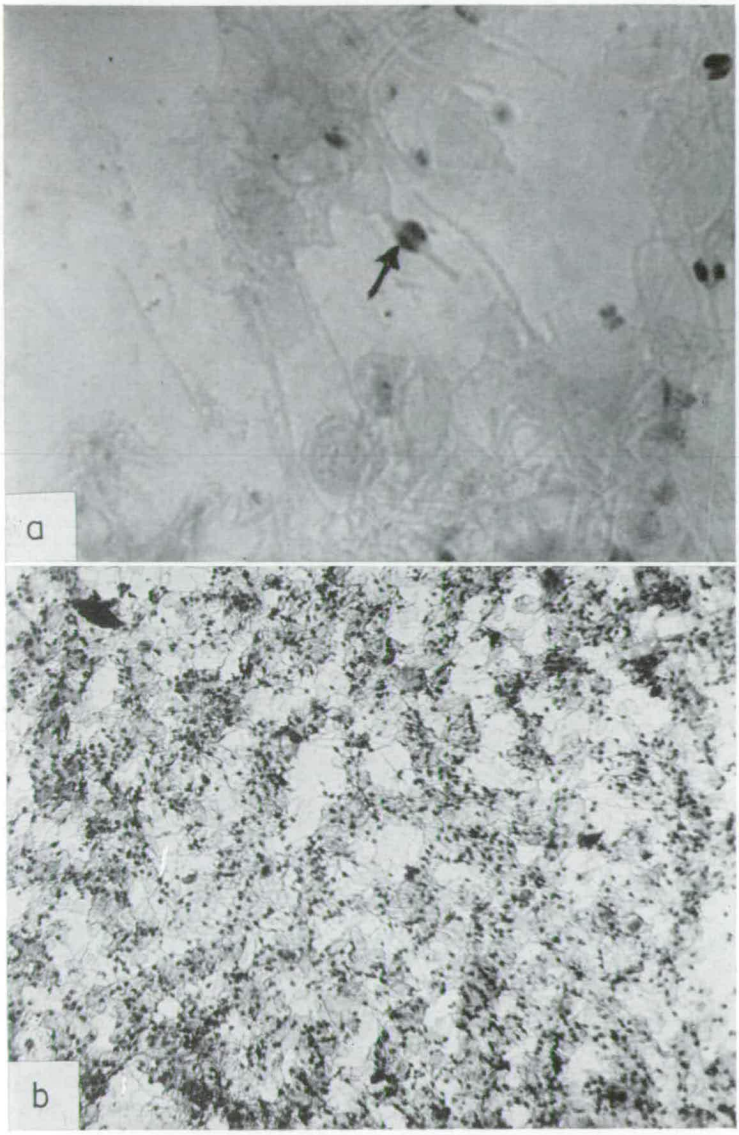

FIG. a. Spermatozoa from the testis and ductuli efferentes of the rabbit showing alkaline phosphatase reaction confined to the cytoplasmic droplet. × 1500.

FIG. b. Spermatozoa taken from the tail of the epididymis of the rabbit showing a mass of cytoplasmic droplets stained for alkaline phosphatase. × 260.

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J. Reprod. Fert. (1972) **29,** 65–69

ALKALINE PHOSPHATASE IN THE CYTOPLASMIC DROPLET OF MAMMALIAN SPERMATOZOA

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Summary. Alkaline phosphatase activity has been examined in ram, rabbit, hamster and rat spermatozoa. Results have shown that the enzyme is restricted to the cytoplasmic droplet in rabbit and ram spermatozoa but that the intensity of reaction is highest in rabbit spermatozoa. The enzyme could not be demonstrated at all in the spermatozoa of the hamster or rat.

It has also been shown that alkaline phosphatase activity in the droplet of rabbit spermatozoa decreases during epididymal transit and that there is a reduction in activity in degenerating spermatozoa. Degeneration in spermatozoa was produced in rabbits by means of artificial cryptorchidism, and after 2 weeks, activity in the droplets was completely lost.

INTRODUCTION

It is still not clear whether the cytoplasmic droplet of the mammalian spermatozoon is simply a remnant of spermatid cytoplasm that is devoid of any special functional significance, or whether it plays a rôle in the nourishment of the spermatozoon as it passes through the epididymis. Migration of the droplet from the neck of the spermatozoon to the end of its mid-piece coincides with the process of sperm maturation, perhaps the most striking morphological change that this cell undergoes during its passage through the epididymis, and yet the droplet seems to have been overlooked by most workers or disregarded as being functionally insignificant. Its structural details have been described (Bloom & Nicander, 1961) but recently only the work of Dott & Dingle (1968) on lysosomal enzymes in bull spermatozoa, and that of Gurya (1963) on cytoplasmic inclusions in the droplet of goat, sheep and buffalo spermatozoa, even hinted at a possible function for the droplet. More recently, Bavdek & Glover (1970) have reported alkaline phosphatase activity in the droplet of rabbit spermatozoa taken from both intact and ligated epididymides and have further suggested that the droplet in this species might be functionally significant.

However, the incidence and distribution of alkaline phosphatase in the spermatozoa of other mammalian species have not hitherto been clarified, and the present work was undertaken in part as an attempt to fill this gap. It was also felt that, by examining alkaline phosphatase activity in maturing and

degenerating spermatozoa, some clue to the enzyme's importance might be obtained.

MATERIALS AND METHODS

Material was obtained for staining from the caput flexure of the epididymis and the cauda epididymidis of four rabbits, six hamsters and six rats immediately after death, and ejaculated spermatozoa were collected from two rabbits by means of an artificial vagina (Walton, 1958). In four rams, samples were obtained from the rete testis, caput flexure and cauda epididymidis as soon as possible after the organs reached the laboratory from the abattoir. Thus, preparation of all epididymal material began within 45 min of an animal's death and in most of the small animals it was prepared within 10 to 15 min.

In each animal, a small piece of the caput flexure was removed and cut into smaller pieces with fine scissors in a watch glass. The fluid that emerged was then diluted with a small amount of normal saline before proceeding with the preparation. Mature spermatozoa were obtained by incising the cauda epididymidis. The contents at this level welled out of the duct except in the hamster where they are relatively viscous, and in the rat where they are cheesy in consistency. In each case, however, fluid from this area was also diluted with saline, and then a drop was taken and examined microscopically to ensure that the spermatozoa were actively motile. Fluid from the rete testis of rams was collected by means of a fine catheter in a similar way to the method described by Setchell, Scott, Voglmayr & Waites (1969) for anaesthetized animals. This was done in order to obtain as clean a sample as possible, largely free from contamination with germinal tissue. This was not possible in the rabbit, but it was found that satisfactory samples could be obtained by incising the dorsal pole of the testis at a point just beneath the initial segment of the epididymis.

Every sample was washed three times in normal saline, centrifuging each time at low speed for about 2 min, in order to remove excess epididymal plasma which might itself contain alkaline phosphatase (Bell & Lake, 1962). The procedure was shown to be absolutely necessary in the golden hamster where the content of alkaline phosphatase in epididymal plasma was so high that, after staining, the spermatozoa were completely obscured on the slides as a result of a generalized reaction.

After the samples were washed, smears were made and these were allowed to dry for a few minutes before being fixed in acetone for about 1 hr at 0° C. The alkaline phosphatase was demonstrated by the use of a modified form of Gomori's method (Culling, 1963), using sodium β-glycerylphosphate as substrate at 37° C and at a pH of 9·5. Controls were incubated in substrate that contained 4 mM-cystine hydrochloride. Different incubation periods were tried but in general, rabbit spermatozoa were incubated at 5, 10 and 20 min, while those of the other species were incubated at $\frac{1}{2}$, 1, 2, 3, 4 and 8 hr.

Spermatozoa were taken from the testis and from different levels of the epididymis to determine the effect of sperm maturation on the distribution of alkaline phosphatase. The fate of the enzyme in degenerating spermatozoa was also investigated, using artificially cryptorchid rabbits. The rabbits were made

cryptorchid by the method previously described by Glover (1959). Spermatozoa were obtained from the epididymis in one animal after 2 days of cryptorchidism, in another after 1 week, and in a third after 2 weeks.

RESULTS

Alkaline phosphatase activity in the cytoplasmic droplet of rabbit spermatozoa was established and confirmed but the reaction was completely negative in the droplet of spermatozoa from the hamster and rat even after as much as 12 hr incubation. A positive reaction was obtained, however, in the droplets of ram spermatozoa, which reacted rather differently from those of rabbit spermatozoa so the two will be considered separately.

Alkaline phosphatase reaction of rabbit sperm droplets

Spermatozoa taken from the testis of the rabbit showed a clear positive reaction in the cytoplasmic droplet after only 5 min incubation. This did not apply to spermatozoa taken from the caput flexure which required 10 min incubation to produce a comparable effect (Pl. 1, Fig. 1). Spermatozoa from the cauda epididymidis (Pl. 1, Figs 2 and 3) and both retained and discarded droplets of ejaculated spermatozoa needed 20 min incubation to give a good reaction. There was no apparent change in reaction, therefore, when the droplet was discarded in a fresh ejaculate. Activity was always restricted to the cytoplasmic droplet except in mature spermatozoa, in which 4 hr incubation elicited a reaction in the mid-piece also.

Alkaline phosphatase reaction of ram sperm droplets

A positive reaction was obtained in ram spermatozoa only after 3 hr incubation or longer. In immature spermatozoa, activity was mostly limited to the cytoplasmic droplet, although there was a very faint reaction in the mid-piece (Pl. 1, Fig. 4). In mature spermatozoa, however, using the same period of incubation, the mid-piece reaction was comparatively intense (Pl. 1, Fig. 5). As with rabbit spermatozoa, there was no indication of any reaction in the acrosome.

Alkaline phosphatase reaction in droplets of degenerating spermatozoa

After 2 days of artificial cryptorchidism in the rabbit, alkaline phosphatase was still clearly detectable in the cytoplasmic droplet, though subjectively it appeared slightly weaker than in control preparations (Pl. 1, Fig. 6). After 7 days, there was a pronounced decrease in the intensity of the reaction (Pl. 1, Figs. 7 and 8) and in samples taken after 14 days of cryptorchidism, it had disappeared altogether except for a very faint reaction in a few droplets (Pl. 1, Fig. 9). It was also noticed that, even after 2 days, the cytoplasmic droplet had ostensibly decreased in size (compare Pl. 1, Figs. 1 and 7).

DISCUSSION

These results show that the cytoplasmic droplets of spermatozoa of the rabbit and ram are rich in alkaline phosphatase. This is especially true in the rabbit

where the droplet is rather larger than in ram spermatozoa. The droplets of rat and hamster spermatozoa show no reaction even after a long period of incubation and, although the possible presence of low levels of the enzyme cannot be entirely discounted, its apparent absence is interesting since, in the epididymis of these species, unlike that of rabbit and ram, alkaline phosphatase is also absent from the microvilli (Maneely, 1955; Nicander, 1957; Linnetz & Amann, 1968; and personal observations).

Wislocki (1950) and Friedlaender & Fraser (1952) claimed to have noted the occurrence of alkaline phosphatase activity in the head of human and ram spermatozoa, respectively, but this finding was not confirmed in the present work. However, the above authors used no controls and also adopted very long periods of incubation (up to 72 hr) which could lead to artifact due to non-specific absorption of the lead content of the stain. There is no record either, in this earlier work, of the spermatozoa having been washed relatively free of plasma. It would seem reasonable to believe, therefore, that, when alkaline phosphatase is present in mammalian spermatozoa, it is mainly concentrated in the cytoplasmic droplet.

Occurrence of activity in the mid-piece is nevertheless of interest, particularly since it appears to increase at this site in association with maturation of the spermatozoa. Moreover, present results indicate a corresponding decrease in activity in the droplet, which suggest that if there is a function involved, it is gradually taken over by the mid-piece. This is perhaps understandable when the droplet is likely to be discarded at ejaculation. The difficulty with histochemical methods of this kind is that some interpretations are bound to be subjective, and Bavdek & Glover (1970) did not have the impression that the intensity of reaction in the droplet decreased when spermatozoa matured. They did not record the incubation times they used, however, and, in the present work, decreased activity during maturation of the spermatozoa was unmistakable.

There is no doubt, moreover, that severely degenerated spermatozoa are virtually devoid of alkaline phosphatase, although a weak reaction was to be seen in some rabbit spermatozoa after 7 days of artificial cryptorchidism. Even during the first week of artificial cryptorchidism, the cytoplasmic droplet of

EXPLANATION OF PLATE 1

Preparations were stained by the modified Gomori's method (see text) and all micrographs were taken at the same exposures and at a magnification of × 1000.

FIG. 1. Normal rabbit spermatozoa from caput epididymidis. 10-min incubation.

FIG. 2. Normal rabbit spermatozoa from cauda epididymidis. 10-min incubation.

FIG. 3. Normal rabbit spermatozoa from cauda epididymidis. 20-min incubation.

FIG. 4. Normal ram spermatozoa from rete testis. 4-hr incubation.

FIG. 5. Normal ram spermatozoa from cauda epididymidis. 4-hr incubation.

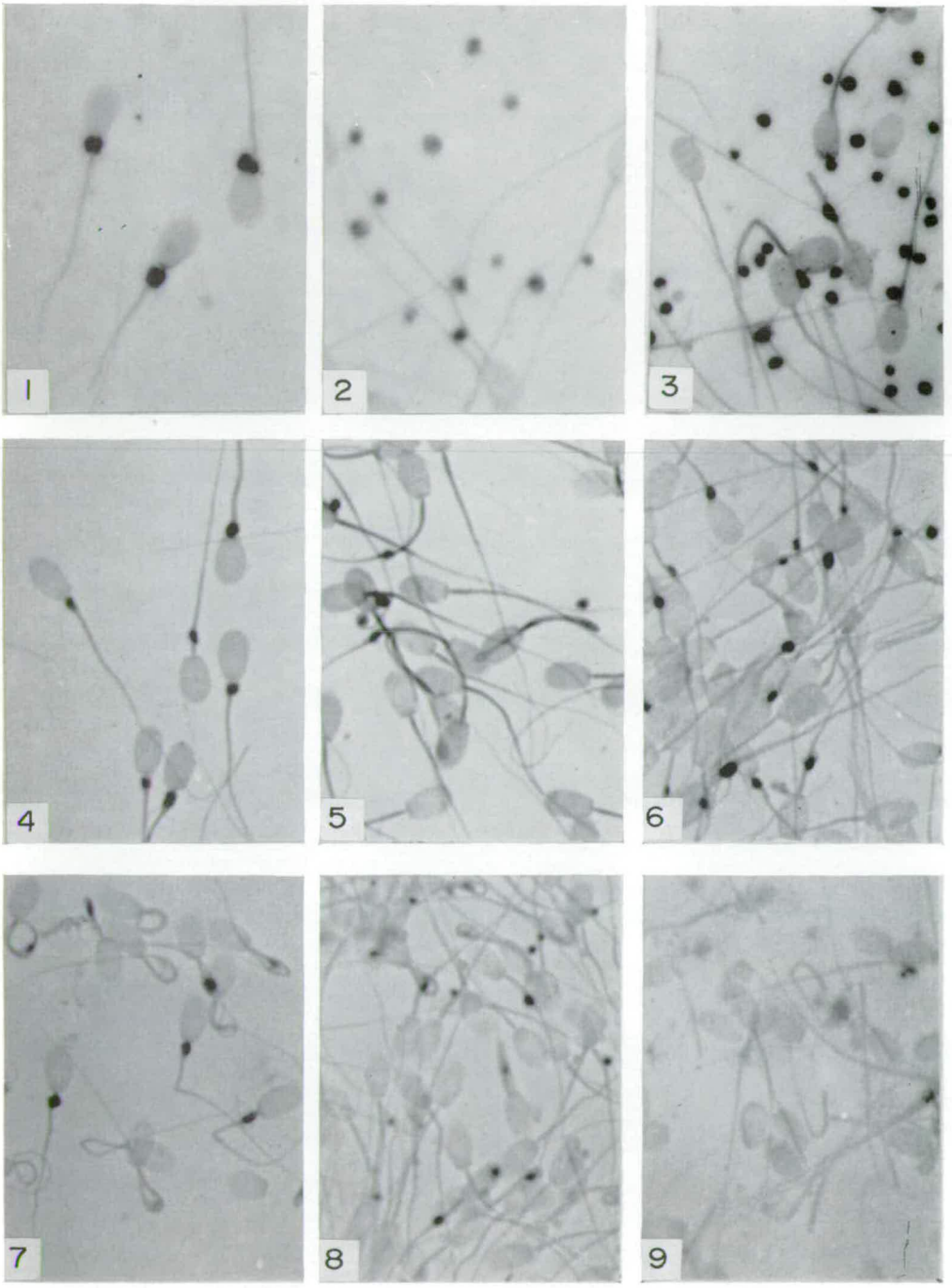
FIG. 6. Spermatozoa from cauda epididymidis of 2-day artificially cryptorchid rabbit. 20-min incubation. Note the retained cytoplasmic droplet.

FIG. 7. Spermatozoa from caput epididymidis of 1-week artificially cryptorchid rabbit. 10-min incubation. Note the reduction in size of the cytoplasmic droplet.

FIG. 8. Spermatozoa from cauda epididymidis of 1-week artificially cryptorchid rabbit. 20-min incubation.

FIG. 9. Spermatozoa from cauda epididymidis of 2-week artificially cryptorchid rabbit. 20-min incubation.

PLATE 1



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rabbit spermatozoa appeared smaller than normal (compare Pl. 1, Figs. 1 and 7) when it was stained for alkaline phosphatase. This effect is difficult to interpret, but illustrates some form of change in the nature of the droplet under these conditions. It is interesting in the light of an earlier finding by Cummins & Glover (1968) that there is a loss of fertilizing capacity during the first few days of artificial cryptorchidism which corresponds to a high proportion of ejaculated spermatozoa retaining their cytoplasmic droplet. This change in the cytoplasmic droplet may be merely a reflection of more widespread damage to the spermatozoa, but it is suggested from accumulated evidence that more attention might be paid to the droplet itself.

The possibility still cannot be discounted that alkaline phosphatase in spermatozoa, concentrated as it is in the cytoplasmic droplet or in the mid-piece, plays a part in the function of the cell by the dephosphorylation and transport of phosphate groups between epididymal plasma and the sperm cell. If it were true, differences in the intensity of reaction between immature, mature and degenerated spermatozoa suggest that the enzyme is of particular significance in the early maturation of spermatozoa in the epididymis.

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Effects of gonadectomy and testosterone replacement on the viability of epididymal spermatozoa in the golden hamster (*Mesocricetus auratus*).

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The effects of androgens on the accessory glands of reproduction, on sexual behaviour and on secondary sexual characteristics are well known, but their influence on epididymal function and consequent sperm viability is less clear. The present paper reports on this aspect of androgenic activity in the golden hamster. The effects of bilateral castration on the viability of epididymal spermatozoa and the prevention of these effects by subcutaneous implants of 25 mg testosterone propionate (Organon Laboratories) are recorded.

In each male animal, mature spermatozoa in the cauda epididymidis were isolated from younger and more immature forms by ligation of the epididymis at a point level with the distal pole of the testis. A further ligature was placed round the ductus deferens to ensure a completely closed system. Animals were then put in one of four groups. The first (Group A), consisted of animals with both testes present, the second (Group B) was where the left testis had been removed, Group C where both testes were removed, and Group D where both testes were removed and subcutaneous implants of testosterone propionate were installed.

In assessing the fertilizing capacity of the spermatozoa each isolated cauda epididymidis was first removed and macerated, and the emergent spermatozoa were suspended in Hanks's solution. Samples of these suspensions were then placed in the uterine horns of oestrous females approximately 6 h before ovulation, after the sperm content had been calculated from counts on a haemocytometer slide. (Estimates of the percentage of live and dead spermatozoa were also previously made from sperm smears after staining with aqueous nigrosin eosin.) Eggs were then obtained by flushing from the uterine tubes 30–36 h after ovulation. They were transferred to slides, prepared as whole mounts and examined under phase contrast. Fertility was then judged as the percentage of eggs that had cleaved and contained a spermatozoon (Austin, 1956).

Results showed that in Groups A, B and D spermatozoa had completely lost their fertilizing capacity by 20–24 days, whereas in Group C animals were completely infertile by 10 or 12 days. Failure to fertilize was not due to a decline in the number of live spermatozoa, because live:dead ratios had not changed appreciably at the time of onset of infertility; nor was it due to a total decrease in sperm numbers for it was always possible to use at least 8 million spermatozoa in each sample. This is well over the minimum required for successful fertilization (unpublished observations). However, motility of the spermatozoa ceased a few days after the development of total infertility. This pattern confirms the observation of Benoit (1926) in mice, of White (1932) in rats, and Benoit (1926) and Moore (1927, 1928) in guinea-pigs, that circulating testicular androgen is required by mature epididymal spermatozoa if they are to exhibit normal motility *in vitro*. It is now clear from the present work, that in

[P.T.O.

the golden hamster they also need such androgen in order to maintain their fertilizing capacity. It appears though, that the presence of only one testis is necessary in order to achieve this.

Histological examination of material showed that while ligation of the epididymis and ductus deferens simply resulted in a preponderance of cuboidal and flattened cells in the cauda epididymidis, the added removal of the testis affected the incidence of so-called holocrine cells (Martan & Risley, 1963). It is difficult to explain this until the functional importance of so-called holocrine cells has been more firmly established, but it is interesting in relation to the problem of sperm survival and certainly seems worthy of further investigation.

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THE INFLUENCE OF THE TESTIS ON THE SURVIVAL OF SPERMATOZOA IN THE EPIDIDYMIS OF THE GOLDEN HAMSTER, *MESOCRICETUS AURATUS*

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Summary. If both testes are removed and both epididymides are allowed to remain *in situ* in the golden hamster, the period of sperm survival in the tail of the epididymis is reduced by about half, since the spermatozoa lose their capacity to fertilize in 10 to 12 days. This contrasts with the 20- to 24-day survival of epididymal spermatozoa when one or both testes are present. It appears that only one testis is needed to ensure the normal survival of spermatozoa in both epididymides. Replacement of testosterone by a subcutaneous implant in bilaterally gonadectomized males resulted in normal sperm survival.

Assessment of the ratio of live to dead spermatozoa showed that the ultimate loss of fertilizing capacity by the spermatozoa in both intact and gonadectomized males was not at first due to an increased incidence of dead spermatozoa in the epididymis, though progressively more dead and degenerated spermatozoa were gradually to be seen in the samples.

The fertilizing capacity of the spermatozoa was lost more rapidly (within 8 days) in gonadectomized animals if they were also hypophysectomized. Administration of HCG to these animals failed to maintain normal viability in the spermatozoa.

A gradual decrease in the occurrence of 'light' cells in the lining tissue of the duct is reported, following gonadectomy.

It is concluded that circulating androgen is unquestionably required for the normal survival of spermatozoa in the tail of the hamster epididymis and that it probably achieves its effect through its influence on the epididymal epithelium. The findings are discussed in relation to the general hormonal control of sperm survival in the epididymis.

INTRODUCTION

It is well known that the epididymis serves to convey maturing spermatozoa from the testis to the ductus deferens, and to store the matured forms in the cauda epididymidis. The spermatozoa cannot be stored here indefinitely, however, and if they are not ejaculated, they gradually lose their capacity to fertilize

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or to produce viable embryos (Hammond & Asdell, 1926; Tesh & Glover, 1969). Temporally, this loss of fertilizing capacity varies between species, although viability is retained for quite some time: in the guinea-pig, for 20 to 35 days (Young, 1929); in the rabbit, for 40 to 50 days (Hammond & Asdell, 1926; Tesh & Glover, 1969); in the mouse, for 10 to 14 days (Snell, 1933); in the rat, for 21 days (White, 1933). By contrast, spermatozoa only survive for a short period in the female tract, and in the rabbit, for example, their fertilizing capacity is lost after about 3 hr (Tesh, 1969).

The special physiological conditions that allow prolonged survival of spermatozoa in the male tract are not precisely known although some factors, such as temperature, are recognized as being involved (Glover, 1960; Cummins & Glover, 1971). The endocrine control of sperm survival is certainly not clear although it is known that the epididymis requires male sex hormone for its growth, structure and functional maintenance (Benoit, 1926; Risley, 1963; Price & Ortiz, 1965). The motility of epididymal spermatozoa *in vitro* is known to be impaired when the testes are removed (Moore, 1928) and it would seem likely that the preservation of fertilizing capacity of the spermatozoa is also androgen-dependent.

Apart from circulating testicular androgen, epididymal spermatozoa have access to two other potential sources of male sex hormone. First, androgens are present in the testicular fluid entering the epididymis and, secondly, the epididymis might itself be capable of androgenesis (Inano, Machino & Tamaoki, 1969; Hamilton, Jones & Fawcett, 1969; Hamilton, 1971), even though species differences in the degree of such activity appear to exist (Frankel & Eik-Nes, 1968, 1970).

The relative importance of these possible sources of androgen in preserving the spermatozoa is unknown and, in the present work, an attempt is made to establish the part played by circulating steroid. The paper first reports on the effects of bilateral gonadectomy and testosterone replacement on the survival of mature spermatozoa in the epididymis of the golden hamster. An initial enquiry has also been made into the rôle of the pituitary in this context.

MATERIALS AND METHODS

Adult, 10-week-old, male and female golden hamsters were kept at a constant temperature of 26° C in a thermostatically maintained room, where a daily period of light from 06.00 to 21.00 hours was alternated with a period of darkness from 21.00 to 06.00 hours by means of an automatic switch. The animals were allowed unrestricted access to food (Diet 41B, Dixon (Ware) & Sons Ltd) and water.

Experimental groups of animals

The experiment was designed so that male animals were divided into seven groups (see Table 1). The first of these consisted of six control animals, where both testes and epididymides were left intact. The first experimental group (Group A) contained twenty-five animals in which both testes were present but the epididymides were ligated. Group-B animals (twenty-eight) comprised

unilaterally gonadectomized males (the left testis was removed but both epididymides were igated), whilst Groups C and D comprised bilaterally gonadectomized males (thirty-one and eighteen animals, respectively) with both epididymides ligated. Group-D animals received a replacement of testosterone (25 mg subcutaneous implant, Organon Laboratories Ltd, London). Thus, the term 'gonadectomy' is to be taken literally in the context of these experiments since the epididymides were always allowed to remain even when the testes were removed.

To test whether the viability of spermatozoa following bilateral gonadectomy was maintained by hypophysial hormones, an additional six gonadectomized and hypophysectomized males were allocated to two groups (E and F). The animals in one of these groups (Group F) received eight daily injections of 100 i.u. HCG and, in each case, animals of Groups E and F were killed after 8 days and the fertilizing capacity of the retained epididymal spermatozoa was determined.

Table 1. Number of male hamsters used in each experimental group at various times

Animal Group	*Days*														
	0	4	5	6	7	8	9	10	11	12	16	18	20	22	24
Controls	6	—	—	—	—	—	—	—	—	—	—	—	—	—	—
A	2	2	—	—	—	2	—	—	—	2	5	3	5	2	2
B	—	4	—	—	—	4	—	—	—	4	4	4	4	—	4
C	2	2	2	2	2	5	5	7	2	2	—	—	—	—	—
D	—	—	—	—	—	—	—	—	—	2	5	5	4	—	2
E	—	—	—	—	—	3	—	—	—	—	—	—	—	—	—
F	—	—	—	—	—	3	—	—	—	—	—	—	—	—	—

Characteristics of Groups (see text): (A) testes present, epididymides ligated; (B) unilateral gonadectomy, epididymides ligated; (C) bilateral gonadectomy, epididymides ligated; (D) bilateral gonadectomy, epididymides ligated, replacement therapy (testosterone); (E) bilateral gonadectomy, hypophysectomy, epididymides ligated; (F) bilateral gonadectomy, hypophysectomy, epididymides ligated and replacement therapy (HCG).

Sperm viability

To test the viability of mature spermatozoa, samples were generally taken from both epididymides of each male and placed into both uterine horns of three or four females. Since the variation between results in different females proved to be negligible, the contents of each cauda epididymidis were placed into a single female only when unilaterally gonadectomized males were used (Group B). In general, when the results were unambiguous, this procedure was carried out using only two males, but the experiments were repeated using several males when more variation was observed in initial results (see Table 1). This explains the variation in the numbers of males used in the different groups.

To determine oestrus in the females, a male and female hamster were placed together in a container between 19.00 and 21.00 hours. If the female displayed lordosis, this was taken as positive evidence that ovulation would occur between

01.00 and 03.00 hours (Ward, 1946; Strauss, 1956; Harvey, Yanagimachi & Chang, 1961).

Spermatozoa were always obtained by macerating the cauda epididymidis in 4 ml Hanks' solution and passing the material through sterile gauze. The motility of the spermatozoa was arbitrarily assessed from examination of the filtrate, and the percentage of live spermatozoa was estimated. For this, the sperm suspension (filtrate) was mixed with an equal volume of aqueous nigrosin–eosin and a smear was made on three separate slides. A total of 250 spermatozoa were counted on the three slides, 100 on each of two and 50 on the third slide. This number was chosen for convenience of calculation and permitted an estimate of variation in the sub-samples to be obtained.

The sperm suspension (0·1 ml) was then diluted to 20 ml with 10% formol saline and the sperm concentration was assessed using a Fuchs–Rosenthal haemocytometer slide. Between 8×10^6 and 16×10^6 spermatozoa were deposited into both uterine horns of female hamsters between 20.00 and 22.00 hours as in the procedure previously described by Yanagimachi & Chang (1961). Each female was anaesthetized with 0·1 ml sodium pentobarbitone (Nembutal, 60 mg/ml, Abbott Laboratories Ltd)/100 g body weight, given intraperitoneally.

The fertilizing capacity of the spermatozoa was then estimated by the examination of tubal eggs flushed from the uterine horns with 0·2 ml Hanks' solution between 30 and 36 hr after ovulation. This was carried out with an ordinary record syringe using a fine needle (gauge-32) inserted into the infundibulum of the uterine tube. The tubes were flushed twice and, after transferring the eggs to a slide, whole mounts were made according to the method described by Chang (1952). The criterion of fertilization was the presence of a decapitate spermatozoon within the vitellus of a cleaved egg.

Recovery of eggs by this means was almost always 100% effective and, on this assumption, the fertilizing capacity was assessed as:

$$\frac{\text{Number of eggs fertilized}}{\text{Number of eggs recovered}} \times 100\%$$

Histological examination

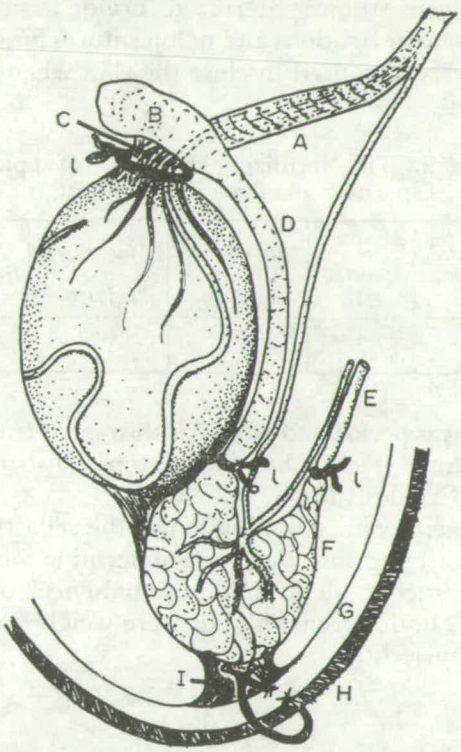
Tissue from each cauda epididymidis was fixed for 24 hr in Bouin's fluid, embedded in paraffin wax, sectioned at 7 μm, stained with Harris' haematoxylin and eosin and mounted in DPX. As part of this examination, the incidence of 'light' cells was determined on material from three males killed at different times after ligation of the duct. The number of these cells was counted for each animal in ten areas of tubule as revealed in a histological section.

Fructose estimations

The effects of gonadectomy and testosterone replacement were also tested by estimating the content of fructose in the seminal vesicle. For this, the seminal vesicles were drained and blotted on filter paper and stored at −20° C until required. The method used to determine fructose was basically the same as that described by Lindner & Mann (1960). Each gland was weighed, homogenized

and then centrifuged at 2900 rev/min for 3 min. Equal volumes (0·25 ml) of 5% zinc sulphate and 0·3 N-barium hydroxide were added to the supernatant and the mixture was recentrifuged. A 1-ml vol. of resorcinol (0·1% in ethanol) and 3 ml 30% HCl were next added to 1 ml of the resulting supernatant. The total mixture was then incubated for 10 min at 90° C, allowed to cool and the percentage of light transmission was determined at a wavelength of 520 nm using a CE 303 grating spectrophotometer (Cecil Instruments, Cambridge). Fructose concentration was expressed as mg/g dry weight of tissue by determining the percentage dry weight of seminal vesicles from animals in each group.

All data obtained in the experiment were analysed by using the simple Student *t* test.



TEXT-FIG. 1. Sites of ligation of the hamster excurrent duct before gonadectomy and suture of the inverted ligamentum testis to the tunica vaginalis and scrotal skin. A, spermatic blood vessels; B, caput epididymidis; C, ductuli efferentes; D, corpus epididymidis; E, ductus deferens; F, cauda epididymidis; G, tunica vaginalis, H, scrotal skin; I, ligamentum testis (inverted); l, ligation sites.

Male gonadectomy, hypophysectomy and ligation of the epididymis

For gonadectomy, the testes of each animal were exposed through a small incision in the scrotum and tunica vaginalis, and were removed after ligation of the spermatic blood vessels and the ductuli efferentes. To do this, a single ligature was appropriately placed close to the surface of the testis so that it came to enclose both the blood vessels and the efferent ductules (see Text-fig. 1), and

left the epididymal vasculature intact. Next, the cauda epididymidis was isolated from the rest of the duct by means of a ligature round the distal part of the corpus epididymidis and round the ductus deferens near to the cauda epididymidis (Text-fig. 1). Whenever possible, ligatures were placed beneath blood vessels so that the circulation to the isolated area was minimally impaired.

Finally, as a precaution, in case the cauda epididymidis might be withdrawn into the abdomen after removal of the gonad, the ligamentum testis was sutured to the tunica vaginalis and the scrotal skin as illustrated in Text-fig. 1. The incisions in the tunica vaginalis and scrotal skin were closed separately with nylon sutures. For all these procedures, the males were anaesthetized with sodium pentobarbitone in the same way as the females, and operations were performed in clean conditions. Sterilized braided silk sutures (size 5/0, Ethicon Ltd) were used for ligations and nylon sutures for the closure of wounds. (Two such sutures were also used to close the skin when testosterone implants were inserted.)

Table 2. The fertilizing capacity of epididymal spermatozoa from intact hamsters

Untreated males	*No. of females/ male*	*Mean no. of eggs recovered*	*Mean no. of eggs fertilized*	*Mean % fertilization rate*
6	4	49·5	48·8	97·7

Hypophysectomy was performed on males through a transauricular approach by means of a Hoffman–Reiter hypophysectomy instrument (Model H-200, H. Neuman & Co., Skokie, Illinois, U.S.A.).

In each case, a macroscopic examination of the sella turcica was performed with the aid of a dissecting microscope to determine whether removal of the hypophysis had been successful. As an additional check on the completeness of the hypophysectomy, both adrenal glands were weighed to determine whether hypertrophy had occurred.

RESULTS

Table 2 shows that, when control males were used, 293 out of a total of 300 eggs (97·8%) were fertilized, and Table 3 shows that spermatozoa retained this high fertilizing capacity for 12 days when isolated in a ligated epididymis (Group A) (Text-fig. 2 and Table 3). This applied even when one testis was removed (Group B) and also when testosterone was administered after bilateral gonadectomy (Group D). In Group-B animals, the viability of the epididymal spermatozoa was maintained equally on either side, so the mean figures for this group in Table 3 are based on pooled data.

Thus, in all three groups, the mean fertilization rate decreased to approximately 55% on the 12th day after operation and, although a steady decline occurred thereafter, differences between Days 16 and 18 and between Days 18

and 20 were not significant. The difference between Day 16 and Day 20 was significant though ($P<0{\cdot}01$), and a drastic effect on fertilizing capacity occurred on and after the 22nd day.

In sharp contrast to these results, the mean fertilizing capacity of epididymal spermatozoa following bilateral gonadectomy began to decline after about 8 days (87·3±4·2%) (Text-fig. 2 and Table 4), and was severely affected after 10 days (32·5±19·9%), although animals varied greatly in their response about this time (Table 4). There was less variation, however, on Days 11 and 12

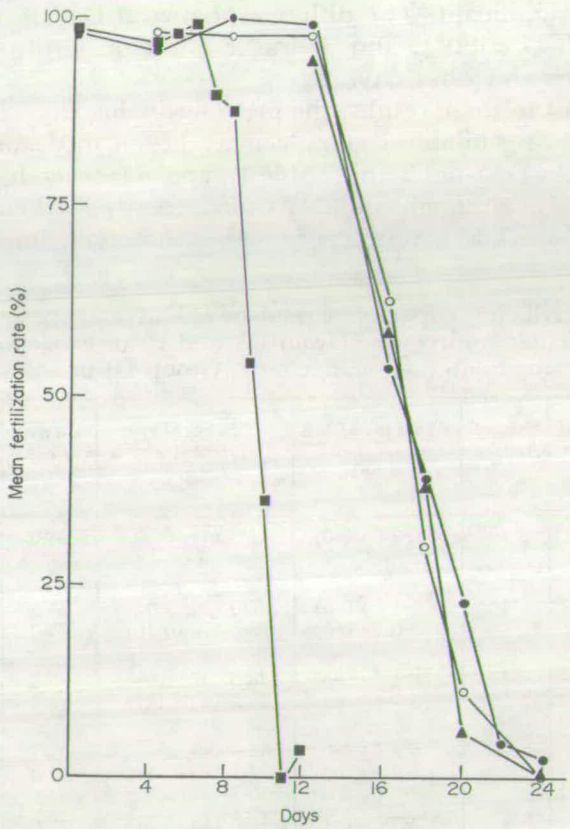
Table 3. The fertilizing capacity of retained epididymal spermatozoa in the presence of testicular androgens (Groups A and B) or exogenous androgens following bilateral gonadectomy (Group D) in hamsters

Days after treatment	*Animal groups*	*No. of females/ male*	*Mean no. of live sperm./uterine horn* $\times 10^6$ (±*S.E.*)	*Mean no. of eggs recovered (with ranges)*	*Mean no. of eggs fertilized*	*Mean % fertilization rate* (±*S.E.*)
0	A	4	6·9 (±0·0)	44·0 (44, 44)	43·0	97·7 (±0·0)
	B	—	—	—	—	—
	D	—	—	—	—	—
4	A	4	4·1 (±0·0)	46·0 (39, 53)	44·5	96·4 (±1·1)
	B	1	5·6 (±0·5)	25·5 (10 to 16)	25·0	97·9 (±1·9)
	D	—	—	—	—	—
8	A	4	8·4 (±0·5)	38·5 (37, 40)	38·5	100·0 (±0·0)
	B	1	5·7 (±0·3)	23·2 (9 to 14)	22·5	97·1 (±2·7)
	D	—	—	—	—	—
12	A	4	6·3 (±1·0)	52·0 (48, 56)	51·5	99·0 (±0·7)
	B	1	6·9 (±0·3)	22·5 (9 to 15)	22·0	97·6 (±2·2)
	D	4	7·6 (±0·0)	36·5 (35, 38)	34·5	94·2 (±4·1)
16	A	4	3·5 (±0·1)	42·8 (38 to 51)	22·6	53·9 (±14·4)
	B	1	3·7 (±0·3)	26·8 (8 to 15)	17·5	62·9 (±12·7)
	D	3 or 4	3·6 (±1·1)	37·8 (36 to 39)	21·6	58·7 (±13·6)
18	A	3 or 4	1·1 (±0·1)	33·3 (32 to 35)	13·3	39·2 (±0·8)
	B	1	6·0 (±0·8)	20·5 (8 to 13)	6·2	30·2 (±7·1)
	D	3 or 4	1·1 (±0·4)	30·6 (23 to 38)	11·8	38·7 (±11·2)
20	A	3 or 4	2·5 (±1·3)	39·0 (31, 47)	10·2	23·0 (±12·1)
	B	1	2·2 (±0·4)	24·5 (9 to 15)	2·7	11·1 (±5·4)
	D	4	2·4 (±0·5)	41·2 (33 to 51)	3·0	6·0 (±4·0)
22	A	4	1·2 (±0·2)	40·5 (40, 41)	2·0	4·8 (±3·5)
	B	—	—	—	—	—
	D	—	—	—	—	—
24	A	4	1·0 (±0·1)	42·0 (40, 44)	1·0	2·4 (±1·7)
	B	1	0·5 (±0·2)	19·7 (6 to 13)	0·0	0·0 (±0·0)
	D	4	1·0 (±0·1)	36·5 (36, 37)	0·0	0·0 (±0·0)

when the adverse effects of removing the testes were unambiguous. When bilaterally gonadectomized animals were hypophysectomized, however, the fertilizing capacity of the spermatozoa was completely lost after 8 days (Group E) and HCG treatment after this operative procedure did not prevent the effect (Group F) (Table 5).

Sperm morphology

The cauda epididymidis of unmated animals always contained a high pro-



TEXT-FIG. 2. The fertilizing capacity of spermatozoa isolated in the hamster cauda epididymidis in the presence of circulating androgens (Groups A, B and D) or after the withdrawal of circulating testicular androgens following bilateral gonadectomy (Group C). ●, Group A; ○, Group B; ■, Group C; ▲, Group D.

Table 4. The fertilizing capacity of retained epididymal spermatozoa in the absence of circulating testicular androgens following bilateral gonadectomy (Group C) in hamsters

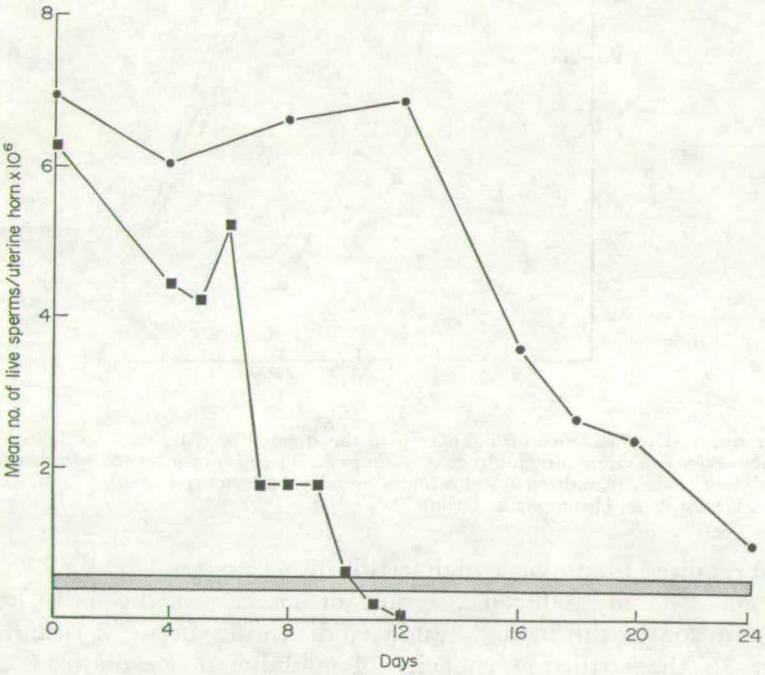
Days after bilateral gonadectomy (Group C)	*Mean no. of live sperm./uterine horn* $\times 10^6$ *(±S.E.)*	*No. of females/ male*	*Total mean no. of eggs recovered (with ranges)*	*Total mean no. of eggs fertilized (with ranges)*	*Mean % fertilization rate (±S.E.)*
0	6·2 (±0·2)	4	51 (50, 52)	50 (48, 52)	98 (±1·4)
4	4·4 (±0·5)	4	46·5 (53, 40)	44·5 (49, 40)	96·2 (±2·7)
5	4·2 (±0·07)	4	46·5 (46, 47)	45·5 (45, 46)	97·8 (±0)
6	5·2 (±1·7)	4	53·5 (54, 53)	53 (54, 52)	99 (±0·7)
7	1·7 (±0·8)	4	37·5 (36, 39)	33·5 (35, 32)	89·6 (±5·4)
8	1·7 (±0·8)	4	47 (45 to 51)	41 (34 to 49)	87·3 (±4·2)
9	1·7 (±0·8)	4	46·6 (46 to 50)	25 (0 to 48)	54·7 (±17·4)
10	0·5 (±0·1)	3 or 4	42·3 (32 to 55)	16·5 (1 to 54)	36·8 (±14·9)
11	0·2 (±0·05)	4	28·5 (25, 32)	0 (0)	0 (±0)
12	0·1 (±0)	4	44 (44, 44)	1·0 (2, 0)	3·4 (±0·7)

portion of effete spermatozoa and they were classified into four groups as follows: (a) intact spermatozoa with a swollen acrosome (Pl. 1, Fig. 2); (b) spermatozoa with a partially detached acrosome (Pl. 1, Fig. 3); (c) spermatozoa bereft of an acrosome (Pl. 1, Fig. 4); and (d) decapitate spermatozoa.

These four categories represent successive stages in the degeneration of non-ejaculated spermatozoa.

Table 5. The fertilizing capacity of retained epididymal spermatozoa following gonadectomy and hypophysectomy (Group D) and HCG treatment (Group F) in hamsters

Days after treatment	*Animal group*	*No. of females/ male*	*Total mean no. of eggs recovered*	*Total mean no. of eggs fertilized*	*Mean % fertilization rate (±S.E.)*
8	D	3	31	0	0 (±0)
8	E	3	33	0	0 (±0)



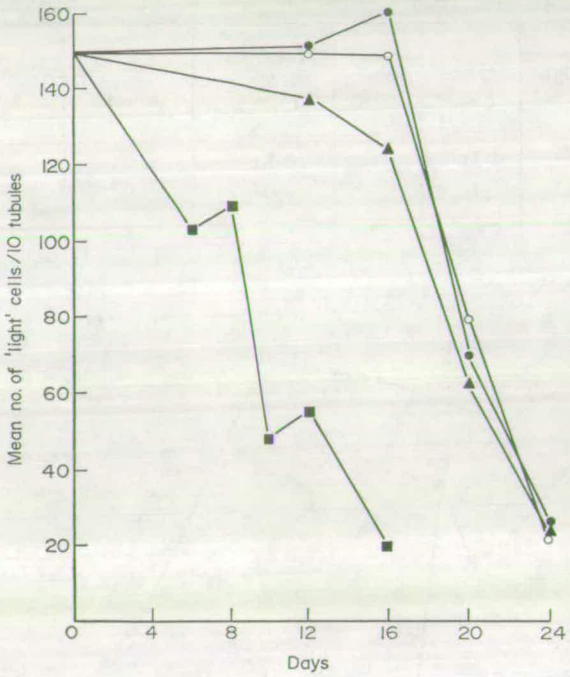
TEXT-FIG. 3. Mean number of live spermatozoa injected/uterine horn in hamsters at various time intervals following treatment: (i) circulating androgen present (Group A: ●); (ii) after the withdrawal of circulating testicular androgens following bilateral gonadectomy (Group C: ■). The hatched bar represents the minimum number of spermatozoa required for maximum fertilization.

Number of live spermatozoa deposited in utero

It appears that when nigrosin–eosin is used to stain hamster spermatozoa, there is almost always some degree of staining irrespective of whether or not the spermatozoa are motile, and only a very few forms are seen to be completely

unstained. However, two distinct types of spermatozoa can be identified as those which are eosinophilic throughout their entire length, and those where only the posterior part of the sperm head is stained (Pl. 1, Fig. 1). We have classified the second of these as 'live' or 'non-eosinophilic' spermatozoa, and the others as 'dead' spermatozoa.

In all animals from Groups A, B or D, the mean number of live spermatozoa had significantly decreased when the cauda epididymidis was isolated for 20 days, but the mean number of live spermatozoa (24×10^6) still exceeded the



TEXT-FIG. 4. The incidence of 'light' cells in the isolated hamster cauda epididymidis in the presence of circulating androgens (Groups A, B and D) or after the withdrawal of circulating testicular androgens following bilateral gonadectomy (Group C). ●, Group A; ○, Group B; ■, Group C; ▲, Group D.

minimum required to support a high fertilization rate (see Text-fig. 3). Although bilateral gonadectomy induced a significant decrease in the mean number of live spermatozoa in the ligated cauda epididymidis between 6 and 10 days (Text-fig. 3), the fertilizing capacity of epididymal spermatozoa following bilateral gonadectomy only began to decline after 8 days (87·3±4·2%; Text-

EXPLANATION OF PLATE 1

Aqueous nigrosin–eosin smears of golden hamster spermatozoa from the cauda epididymidis.

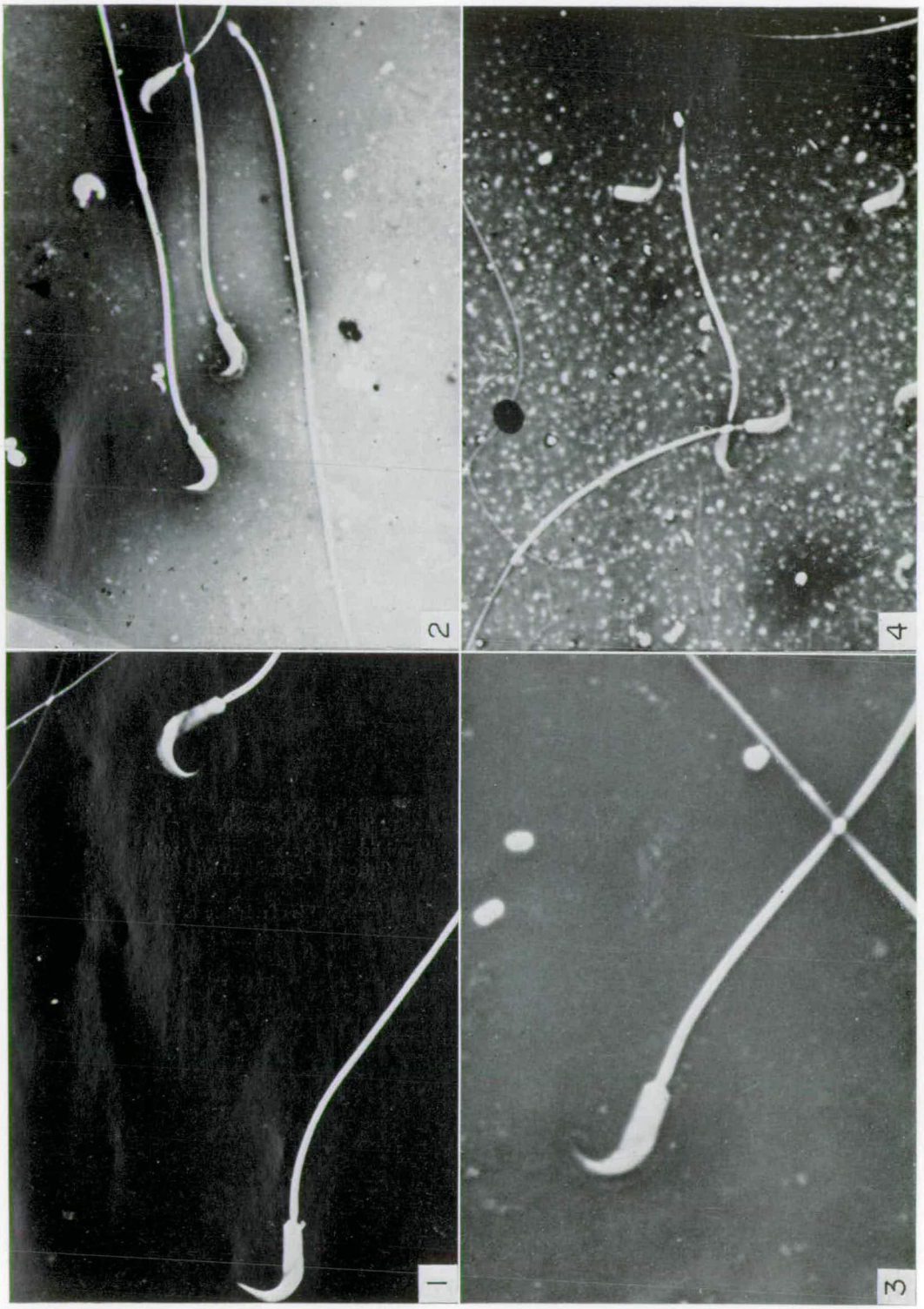
FIG. 1. Live unstained and partially stained spermatozoa. ×640.

FIG. 2. Intact dead spermatozoon with a swollen acrosome (centre). ×480.

FIG. 3. Dead spermatozoon with a ruptured acrosome. ×800.

FIG. 4. Dead spermatozoon bereft of an acrosome. ×480.

PLATE 1



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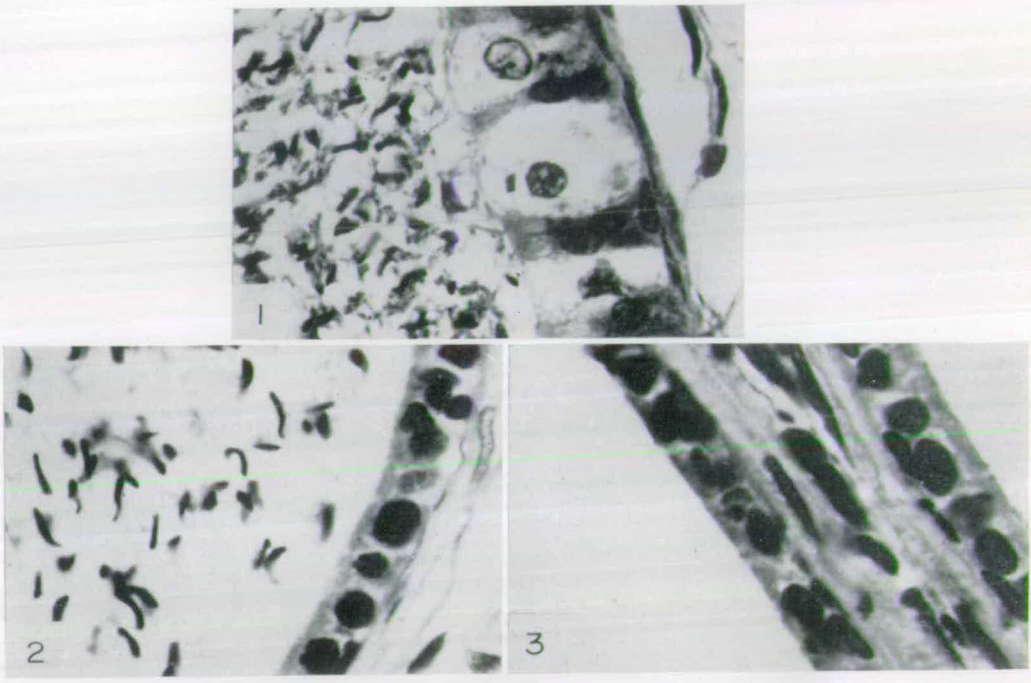

FIG. 1. Tubular epithelium from the cauda epididymidis of an intact golden hamster. Note high columnar principal cells interspersed with 'light' cells. × 220.

FIG. 2. Tubular epithelium from the ligated cauda epididymidis of a hamster in which both testes were left *in situ* (Group A, 20 days). Note the flattened epithelium and the absence of 'light' cells. × 170.

FIG. 3. Tubular epithelium from the ligated cauda epididymidis of a bilaterally gonadectomized hamster. × 170.

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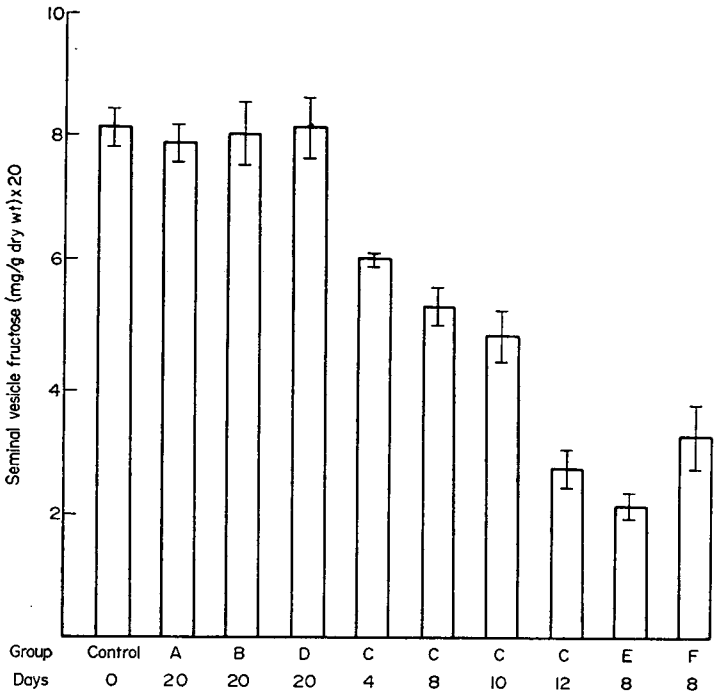

TEXT-FIG. 5. The mean concentration of fructose in seminal vesicles of the seven groups of hamsters used in the study. (For an explanation of the characteristics of Groups A to F, see text.)

Table 6. Seminal vesicle fructose contents at various times under different experimental conditions (Groups A to F) in hamsters

Animal group	*Days*						
	0	4	8	10	12	16	20
Controls	8·11 (±0·3)	—	—	—	—	—	—
A	—	—	—	—	—	—	7·8 (±0·3)
B	—	—	—	—	—	—	8 (±0·5)
C	—	6·02 (±0·2)	5·3 (±0·3)	4·8 (±0·4)	2·7 (±0·24)	—	—
D	—	—	—	—	—	—	8·1 (±0·5)
E	—	—	2·1 (±0·2)	—	—	—	—
F	—	—	3·2 (±0·5)	—	—	—	—

Mean seminal fructose in mg/g dry wt (±S.E.).
For characteristics of Groups, see footnote to Table 1.

fig. 2 and Table 4). This was not due to a paucity of live spermatozoa since the mean number of live spermatozoa deposited between 8 and 10 days after bilateral gonadectomy exceeded the minimum number ($0{\cdot}5 \times 10^6$) necessary to ensure maximum fertilization (see Text-fig. 3).

Histological effects

Examination of material from animals in Groups A, B and D revealed that, within 20 to 24 days, isolation of the cauda epididymidis resulted in flattening of the principal cells of the tubular epithelium and a gradual disappearance of so-called 'holocrine' cells, which are conspicuously interspersed between the principal cells of the epithelium (Pl. 2, Fig. 1). The incidence of these cells decreased significantly ($P<0{\cdot}001$) between 16 and 20 days in Groups A, B and D, and between 8 and 12 days following bilateral gonadectomy (Text-fig. 4). In each case, this decrease paralleled a loss of fertilizing capacity in the spermatozoa.

Fructose concentration of seminal vesicles

The results of these estimations (Text-fig. 5 and Table 6) show that in animals from Groups A, B or D, there was no significant change in the mean concentration of fructose in the seminal vesicles even after 20 days. In the animals from Group C, the fructose concentration decreased significantly after only 4 days, even though the spermatozoa retained a high fertilizing capacity for 8 days (Text-fig. 2). Later, both fructose concentration and fertilization rate decreased precipitously, and, within 12 days of removal of the gonads, they were very low indeed (Text-figs 5 and 2, respectively). The results from animals in Groups E and F, however, show that vesicular fructose was significantly lower ($P<0{\cdot}001$) after 8 days of hypophysectomy and gonadectomy than after gonadectomy alone.

DISCUSSION

It is well known that epididymal spermatozoa *in vitro* lose their motility twice as quickly if the testes have been removed. This has been shown in guinea-pigs (Benoit, 1926; Moore, 1927, 1928), rats (Moore, 1928; White, 1933) and mice (Benoit, 1926), but the present work has demonstrated that the maintenance of the fertilizing capacity of epididymal spermatozoa is also very dependent upon the presence of circulating androgen. After gonadectomy, spermatozoa were incapable of fertilization after about 10 or 12 days, compared to 20 to 24 days in intact animals. It appears, moreover, that only one testis is needed to preserve the spermatozoa because, irrespective of which testis is removed, the spermatozoa in both epididymides retain their fertilizing capacity for the normal period. If both testes are removed, the normal period of sperm survival may be ensured by the administration of exogenous testosterone.

Correspondingly, the morphology of the cells lining the cauda epididymidis seems to be extremely sensitive to circulating androgen, and all the indications are that mature spermatozoa depend, for the preservation of their fertilizing capacity, upon the epididymal cells and that these in turn are dependent on

circulating androgen. Voglmayr, Waites & Setchell (1966), White & Hudson (1968) and Appelgren (1969) have all discussed the subject of androgen and epididymal fluid but it is still not certain whether significant amounts of steroid are present in luminal fluid at the level of the cauda epididymidis. Any androgen that might be present must, in the light of present results, be regarded as less vital than systemic androgen for sperm preservation because unless the cells of the epididymis, which are dependent on circulating androgen, are functioning normally, the spermatozoa cannot retain their fertilizing capacity for the normal period.

Nevertheless, Mounib (1964) and, more recently, Murdoch, White & Seamark (1970) have suggested that testosterone might suppress the oxidative metabolism of spermatozoa in the male reproductive tract and that this might be important in preserving their viability. The suggestion receives some support from the work of Ericsson, Cornette & Buthala (1967) which showed that epididymal spermatozoa will bind labelled testosterone *in vivo*. Ejaculated spermatozoa from a wide variety of species are known to be capable of metabolizing testosterone and other sex steroids (Scott, Wales, Wallace & White, 1963; Seamark & White, 1964; Hathaway, 1965). If, however, epididymal spermatozoa need androgen in the luminal fluid in order to survive, then the bulk of it must be produced or transferred from the bloodstream by the epididymal cells.

The reason why epididymal spermatozoa soon become degraded after removal of the testes is unclear and requires investigation. In this context, it is of interest that, in the present work, loss of fertilizing capacity by the spermatozoa was always accompanied by a decrease in the incidence of 'light' cells or so-called 'holocrine' cells (Malone & Bower, 1962; Martan & Risley, 1963a, b) in the epididymal epithelium. It would be premature to suggest that the two events are related, but Martan & Risley (1963a, b) have suggested that renewal of these cells is both androgen- and pressure-dependent. In the present work, all spermatozoa were obtained from an isolated cauda epididymidis and in these circumstances, the duct swells and the epithelium becomes flattened, suggesting fluid accumulation and increased pressure in the lumen. Disappearance of these cells, therefore, may be nothing more than an artifact induced by the experimental procedure, but the influence of contained spermatozoa upon the cells lining the epididymis would seem to warrant further scrutiny.

Implicit in the rôle of the testes in sperm survival is some activity on the part of the pituitary, and present results have shown that this is not entirely a question of the testicular–hypophysial relationship. It seems that the pituitary exerts its own specific influence on sperm survival since fertilizing capacity in the spermatozoa is lost sooner if animals are hypophysectomized in addition to having their testes removed.

Recently, Goldman & Porter (1970) have shown that, in hamsters, the circulating titre of LH increases tenfold after 10 days of castration. Doubtless, this increase is due to the withdrawal of testosterone and in guinea-pigs, Resko (1970) showed that plasma testosterone had completely disappeared within 6 hr of castration. In our experiments, there was a significant reduction in the fructose content of the seminal vesicles 4 days after castration, and since seminal

fructose is a sensitive indicator of circulating testicular androgen (Mann, 1964), it appears that in hamsters also, testosterone declines very rapidly when the testes are removed. An increase in the level of LH might thus be predicted with the possibility that it might prolong the survival of spermatozoa to some extent. It might operate by stimulating androgenesis in the epididymis (see Hamilton, 1971) or androgen output by the adrenal (see Bullock & New, 1971) but the failure of HCG to prolong sperm survival should be noted. The observation that seminal fructose declined more rapidly after hypophysectomy and gonadectomy than after gonadectomy alone does suggest that a pituitary-dependent extragonadal source of androgen might be involved in sperm survival.

It is now important to determine precisely how circulating androgen produces the right conditions for the optimal survival of spermatozoa in the epididymis.

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THE EFFECTS OF HYPOPHYSECTOMY AND THE PARADOXICAL EFFECT OF HUMAN CHORIONIC GONADOTROPHIN ON THE VIABILITY OF HAMSTER SPERMATOZOA

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SUMMARY

Spermatozoa in the ligatured cauda epididymidis of golden hamsters were tested for their fertilizing ability 12 days after hypophysectomy or after hypophysectomy and treatment with testosterone or human chorionic gonadotrophin (HCG). Fertility trials showed that the mean fertilization rate was reduced to approximately 65% of the control level, which contrasts sharply with the previously reported effects of castration, which led to the infertility of spermatozoa within 12 days as a result of androgen withdrawal.

The present study showed that loss of fertilizing ability in hypophysectomized animals was also due to a decrease in circulating androgens since treatment with testosterone or HCG (10 or 40 i.u./day) prevented the adverse effect of hypophysectomy. However, there was some indication that sperm survival might also be directly or indirectly dependent, in part, on hypophysial hormones. While daily doses of HCG (10 or 40 i.u./day) maintained fertilizing ability in hypophysectomized animals, treatment with intermediate doses (especially 20 i.u./day) reduced the mean fertilization rate to 40·8%. This paradoxical effect of HCG on sperm survival was shown not to be mediated by the adrenal glands: the magnitude of the effect was related to the amount of testicular tissue present. Thus, when only one testis was present the adverse effect of 20 i.u. HCG/day was reduced by approximately half whereas the spermatozoa retained a high fertilizing ability in animals that were both castrated and hypophysectomized and treated with testosterone. It is suggested that the biphasic effect of HCG is due to the peripheral conversion of limited but significant amounts of circulating testicular androgens into oestrogens which antagonize the effect of testosterone in the epididymis. Attention is drawn to the possibility that specific dose levels of HCG might also be detrimental to sperm survival in the treatment with gonadotrophins of infertility in men.

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The present work provides additional support for the hypothesis that the viability of epididymal spermatozoa is primarily dependent on circulating testicular androgens.

INTRODUCTION

A relationship between the pituitary gland and the gonads was first suggested when Crowe, Cushing & Homans (1909) reported involution of the reproductive organs after hypophysectomy in male dogs. Aschner (1912) and Camus & Roussy (1920) subsequently confirmed these findings but considered that inadvertent injury to the brain was partly responsible for the observed involution of the reproductive tract. Smith's (1930) parapharyngeal approach to hypophysectomy in the rat avoided the likelihood of brain damage and produced similar results, which have been subsequently confirmed on numerous occasions in several unrelated species (see Rowlands & Parkes, 1966).

Hamster spermatozoa in the cauda epididymidis only retain their fertilizing capacity if circulating androgen is present (Lubicz-Nawrocki & Glover, 1973). Since the level of androgen is related to interstitial cell-stimulating hormone (ICSH) secretion (Davidson, 1966; Hall, 1970), the experiments reported below were designed to discover the effect of hypophysectomy and replacement therapy (using testosterone and human chorionic gonadotrophin (HCG)) on the viability of epididymal spermatozoa.

MATERIALS AND METHODS

Adult (10 weeks old) male and female hamsters were maintained at an environmental temperature of approximately 26 °C and illuminated from 06.00 h to 21.00 h. They received diet 41 B (Ware & Sons Ltd) and water *ad libitum*.

Each group comprised male hamsters (95–100 g) in each of which the cauda epididymidis was isolated from the rest of the duct by ligatures around the corpus epididymidis and the proximal part of the ductus deferens (see Lubicz-Nawrocki & Glover, 1973). For this, animals were anaesthetized with an intraperitoneal injection of 0·1 ml sodium pentobarbitone (Nembutal, 60 mg/ml, Abbott Laboratories Ltd).

The first experimental group (group A) comprised five hypophysectomized animals with intact testes.

Twenty-three males were hypophysectomized to test whether the adverse effect of hypophysectomy on sperm survival could be prevented by treatment with testosterone or HCG. Three male animals (group B) received subcutaneous implants of 25 mg testosterone (Organon Laboratories Ltd) immediately after hypophysectomy. The remaining 20 hypophysectomized animals were divided into four equal groups: animals in group C received 10 i.u., group D 20 i.u., group E 30 i.u. and group F 40 i.u. of HCG (Chorulon, Organon Laboratories Ltd). Half the dose was injected subcutaneously in the morning and half in the evening.

Since 20 i.u. HCG/day proved detrimental to the viability of spermatozoa, it was decided to test whether this was indirectly mediated through the adrenal glands. Three animals (group G) were hypophysectomized, bilaterally adrenalectomized and treated with 12 daily doses of 20 i.u. HCG in the way described above. These animals were maintained in good health by the addition of sodium chloride (0·9%, w/v) to the drinking water.

Adrenalectomy failed to prevent the adverse effect of HCG on sperm viability. In order to test whether this effect was mediated by the testes, hypophysectomized animals in group H ($n = 3$) were unilaterally gonadectomized whereas three hypophysectomized animals in a further group (group I) were bilaterally gonadectomized and immediately implanted with testosterone. Animals of both groups received 12 daily doses of 20 i.u. HCG, as described above. Animals ($n = 3$) in the control group received no treatment after ligation of the epididymis. The animals in each group were killed 12 days after initiation of the experiments and the fertilizing ability of the epididymal spermatozoa was tested (see below). A 12-day treatment period was selected so that the results would be comparable with the results of previous experiments (Lubicz-Nawrocki & Glover, 1973).

The viability of spermatozoa

The fertilizing ability of epididymal spermatozoa was assessed as previously described by Lubicz-Nawrocki & Glover (1973). Both epididymides were macerated in 4 ml Hanks' solution and approximately 12×10^6 spermatozoa were injected into both uterine horns of three oestrous females. Eggs were flushed from the uterine tubes between 30 and 36 h after ovulation and whole mounts were prepared by the method of Chang (1952). The eggs were examined by phase-contrast microscopy and fertilization was determined by the presence of a headless spermatozoon within the vitellus of a cleaved egg (Austin, 1956). Since almost all ovulated eggs were recovered, fertilizing ability (%) was assessed as: $100 \times$ (no. of eggs fertilized)/(no. of eggs recovered).

The concentration of spermatozoa injected into the uterine horns was determined from haemocytometer counts. The percentage of live spermatozoa was estimated by counting the number of partially stained cells in smears of sperm suspensions stained with aqueous nigrosin–eosin (Lubicz-Nawrocki & Glover, 1973). Since the concentration of spermatozoa was known, it was possible to calculate the number of live spermatozoa injected into each uterine horn.

Gonadectomy, hypophysectomy and adrenalectomy

The procedures for gonadectomy and hypophysectomy were the same as previously described by Lubicz-Nawrocki & Glover (1973). After the hypophysectomized animals had been killed, the sella turcica was examined for pituitary remnants with a dissecting microscope. Both testes and adrenal glands from these animals were weighed to determine whether atrophy had occurred. Adrenalectomy was performed by cautery through a dorsal skin incision.

Histological observations

Testes from each hypophysectomized animal were fixed in Bouin's fluid. Sections were stained with Harris's haematoxylin and eosin and mounted in DPX (dibutylphthalate xylene).

Measurement of fructose in seminal vesicles

As a further test of the effects of hypophysectomy and hormonal replacement, the concentration of fructose in the seminal vesicles was determined by a slight modifi-

cation (Lubicz-Nawrocki & Glover, 1973) of the method originally described by Lindner & Mann (1960).

All data were analysed by Student's *t*-test.

RESULTS

Table 1 shows that when spermatozoa from the control male hamsters were used, 85 out of 87 eggs (97·5 ± 0·9 %) were fertilized by epididymal spermatozoa. However, 12 days after hypophysectomy (group A) the mean fertilization rate was 66·4 ± 6·7 %.

Table 1. *The fertilizing ability of epididymal spermatozoa after hypophysectomy and hormonal replacement in hamsters* (*means* ± S.E.M.)

Group no.	Treatment	Fructose concn in seminal vesicles (20 × mg/g dry wt)	10^6 × no. of live spermatozoa/ uterine horn	Mean no. of eggs recovered	Mean no. of eggs fertilized	Fertilization rate (%)
Controls	None	8·1 ± 0·3	4·9 ± 0·6	29·0	28·3	97·5 ± 0·9
A	Hypophysectomy	6·9 ± 0·3	3·7 ± 0·8	28·8	19·0	66·4 ± 6·7
B	Hypophysectomy and testosterone	7·4 ± 0·2	3·1 ± 0·3	28·0	27·0	98·7 ± 0·5
C	Hypophysectomy and 10 i.u. HCG	8·4 ± 0·6	6·0 ± 0·6	24·5	23·0	94·7 ± 4·0
D	Hypophysectomy and 20 i.u. HCG	8·4 ± 0·3	3·7 ± 0·6	32·8	17·6	40·8 ± 10·4
E	Hypophysectomy and 30 i.u. HCG	8·7 ± 0·6	8·3 ± 0·3	31·7	16·7	54·2 ± 5·4
F	Hypophysectomy and 40 i.u. HCG	8·5 ± 0·5	7·0 ± 0·5	28·0	26·6	95·4 ± 4·0
G	Hypophysectomy and adrenal-ectomy and 20 i.u. HCG	8·4 ± 0·2	6·1 ± 0·9	30·6	11·6	35·7 ± 15·3
H	Hypophysectomy and unilateral gonadectomy and 20 i.u. HCG	9·0 ± 0·9	7·5 ± 0·7	34·2	28·5	85·0 ± 7·0
I	Hypophysectomy and bilateral gonadectomy and testosterone and 20 i.u. HCG	7·4 ± 0·6	6·3 ± 0·5	31·3	30·6	98·0 ± 4·0

HCG = human chorionic gonadotrophin.

This decrease in fertilization rate was prevented in animals that received testosterone (group B) or daily injections of 10 or 40 i.u. HCG (groups C and F, respectively), but with intermediate doses (20 or 30 i.u. HCG) fertilizing ability was lower than after hypophysectomy alone (Table 1). The concentration of fructose in the seminal vesicles of these animals remained high and there was no relationship between the number of live spermatozoa injected and the fertilization rate.

Similar effects on fertilizing ability were obtained when 12 daily doses of 20 i.u. HCG were injected into animals which were both hypophysectomized and adrenal-

ectomized (Table 1). However, in animals which had been both hypophysectomized and unilaterally gonadectomized (group H) and treated daily with 20 i.u. HCG, the spermatozoa retained a high fertilizing ability for 12 days ($85 \pm 7\%$); daily treatment with 20 i.u. HCG in animals receiving testosterone replacement after hypophysectomy and bilateral gonadectomy (group I) had no adverse effect on sperm viability (Table 1).

Twelve days after hypophysectomy the weights of the testes and adrenal glands were reduced significantly ($P < 0{\cdot}001$) ($2{\cdot}8 \pm 0{\cdot}1$ g *v.* $1{\cdot}6 \pm 0{\cdot}2$ g, and $180 \pm 3{\cdot}0$ mg *v.* $134{\cdot}4 \pm 7{\cdot}1$ mg, respectively). When the testes were examined histologically the seminiferous tubules were seen to be aspermic 12 days after hypophysectomy but the Leydig cells appeared histologically normal (Plate, figs 1 and 2).

Table 1 shows that hypophysectomy significantly reduced the mean fructose concentration in the seminal vesicles ($P < 0{\cdot}05$) whereas treatment with testosterone or HCG maintained it at the control level.

DISCUSSION

Hypophysectomy is less detrimental to sperm survival than bilateral gonadectomy (Lubicz-Nawrocki & Glover, 1973). Whereas the latter procedure resulted in complete loss of viability within 12 days, the mean fertilization rate only fell to approximately 66% 12 days after hypophysectomy. As with gonadectomy, however, the loss of fertilizing ability in hypophysectomized animals is primarily due to withdrawal of circulating testicular androgens since spermatozoa in hypophysectomized animals retained a normal fertilizing capacity after treatment with testosterone or certain doses of HCG (10 or 40 i.u./day).

Although in the present work the sella turcica was never examined histologically, the evidence from macroscopic examination ($\times 20$) and of testicular regression and atrophy of the adrenal glands strongly suggests that the hypophyses had been successfully removed on each occasion. Nevertheless, it is feasible that a low level of ICSH secretion persisted after the removal of the main pituitary mass since, in contrast to the rat, the hypophysis in the hamster is relatively compressed, with lateral extensions closely associated with nearby blood-vessels and nerves (Hanke & Charipper, 1948; Serber, 1958, 1961).

Whereas spermatozoa retained a high fertilizing capacity in hypophysectomized animals treated daily with 10 or 40 i.u. HCG, intermediate doses, especially 20 i.u./day, seemed to impair the fertilizing ability of spermatozoa. This effect with 20 i.u. HCG was reduced after unilateral gonadectomy and was completely absent in animals which were both bilaterally gonadectomized and hypophysectomized and which received testosterone implants.

Although the adverse effect of HCG on sperm survival was mediated through the testes and not through the adrenal glands, the precise mechanism of this response is not clear. At present, there is no direct evidence to suggest that either HCG or ICSH act on the seminiferous tubules. It is generally accepted that these hormones exert their main effects on the Leydig cells (Hall & Eik-Nes, 1962; Eik-Nes, 1970; Hall, 1970). It seems most likely, therefore, that the detrimental effect of HCG on sperm survival is related to the enhanced secretory activity of the Leydig cells. These

results, therefore, may be due to a paradoxical effect of androgens whereby a slight increase (within certain levels) in circulating testosterone is detrimental to sperm survival. Such paradoxical effects are well known in the response of various target organs to progesterone and oestrogen (Burrows, 1949).

The paradoxical effect of HCG on sperm survival might be produced by the peripheral conversion of testosterone to oestrogen (Steinach & Kun, 1937; Callow, Callow & Emmens, 1939; Nathanson & Towne, 1939; Epstein, Raheja, Frow & Morse, 1966), causing an inhibition of the effect of testosterone on the epididymis at certain critical ratios. This suggestion is supported by the results of an experiment in which oestradiol benzoate and testosterone were administered simultaneously to intact hamsters (C. M. Lubicz-Nawrocki, in preparation). It is interesting to note, also, that HCG treatment of normal men or those suffering from testicular hormone deficiency (Maddock & Nelson, 1952) induces hyperplasia of the Leydig cells and increases the urinary excretion of oestrogens (Maddock & Nelson, 1952; Paulsen, 1965).

These findings may be of clinical interest since both HCG and human menopausal gonadotrophin (HMG) are necessary to restore normal spermatogenesis in cases of infertility in hypogonadal men caused by malfunction of the pituitary gland. Failure to remedy infertility in these cases may stem from the use of excessive amounts of HCG. In relation to body weight, the daily administration of 10 i.u. HCG to hypophysectomized hamsters would correspond to approximately 500 i.u./70 kg in man so that adverse effects in men with pituitary malfunction might be expected with daily doses of 1000–1500 i.u. HCG. The relatively few successful treatments of infertility in men involved mean daily doses of less than 750 i.u. HCG (Polishuk, Palti & Laufer, 1967; Johnsen & Christiansen, 1968; Paulsen, 1968; Granville, 1970) and recently Paulsen (1968) described the successful treatment of infertility in a man treated with HMG and HCG (approximately 500 i.u./day). After this the dose of HCG was increased above 1000 i.u./day but the wife of this patient conceived again only after the HCG was reduced to the previous level. Gemzell (1970) considers it advisable to use small doses of HCG in the treatment of human infertility since high doses cause peritubular fibrosis and decrease sperm production (Nelson & Heller, 1948; Maddock & Nelson, 1952; Martin, 1967).

In conclusion, the present results support the concept that withdrawal of circulating androgen is detrimental to sperm survival and that treatment of hypophysectomized animals with certain doses of HCG will impair the fertilizing capacity of spermatozoa in the presence of normal levels of circulating testosterone (using seminal vesicular fructose as an indicator of the circulating level of androgen).

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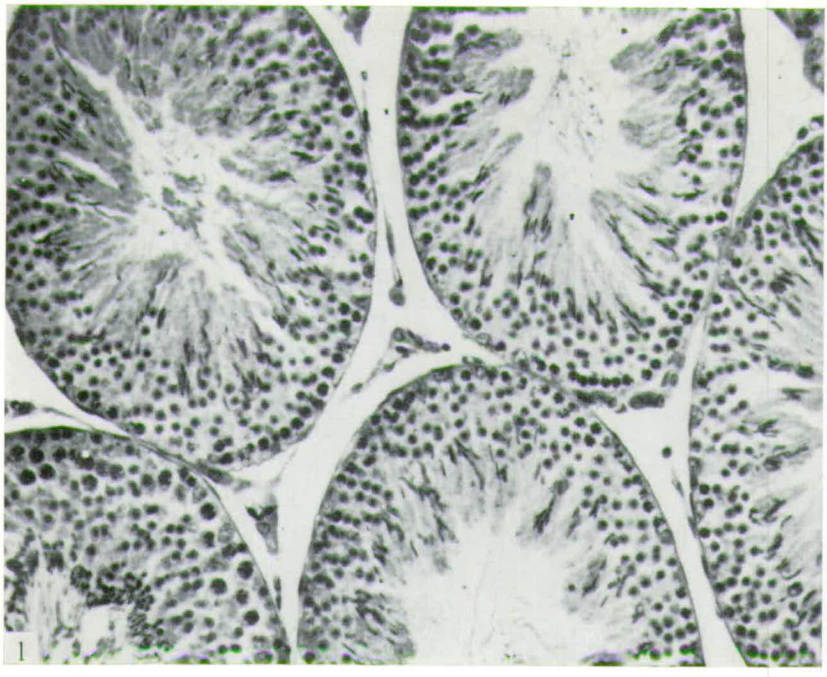
DESCRIPTION OF PLATE

Sections through the testes of hamsters. Haematoxylin and eosin. (× 80.)

Fig. 1. Testis from an intact, untreated hamster.

Fig. 2. Testis 12 days after hypophysectomy. Note aspermic tubules but histologically normal Leydig cells.



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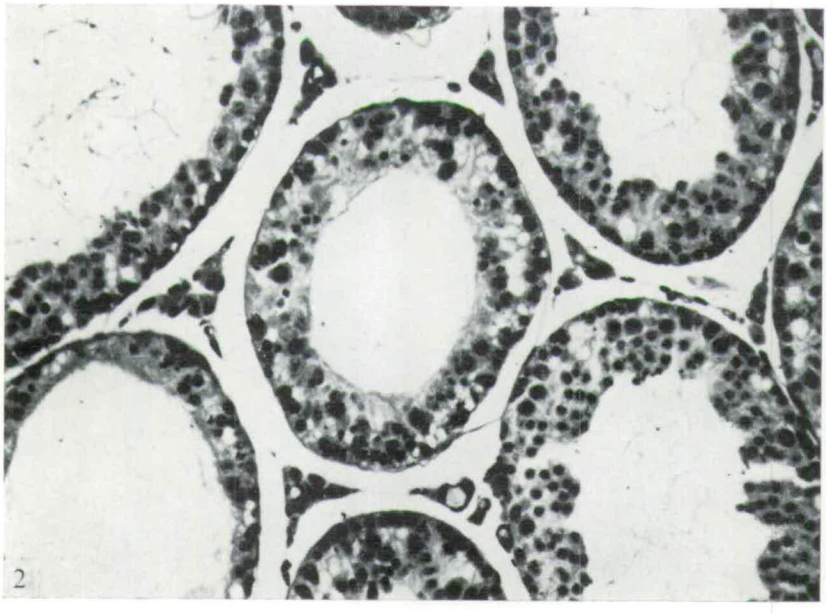

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J. Reprod. Fert. (1973) **34**, 331–339

THE INFLUENCE OF 17α-METHYL-B-NOR-TESTOSTERONE (SK & F 7690) ON THE FERTILIZING ABILITY OF SPERMATOZOA IN HAMSTERS

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Summary. The effect of 17α-methyl-B-nortestosterone (SK & F 7690) on spermatozoa in the isolated cauda epididymidis has been investigated in hamsters and it was found that the survival of the spermatozoa was impaired after treatment with this anti-androgen, which thereby caused rapid loss of fertility. Tests were carried out with entire animals so that endogenous androgens were present, and then with castrates in which exogenous androgens were administered by implant.

In castrated animals given testosterone, the anti-androgen competitively inhibited the stimulatory effects of androgens in the cauda epididymidis and impaired the fertilizing capacity of contained spermatozoa after 12 days. Loss of fertilizing capacity was not related to an increase in the proportion of dead spermatozoa and occurred before the live/dead ratio changed significantly. The anti-androgen appeared to impair an androgen-dependent aspect of epididymal function concerned with maintenance of the fertilizing capacity of contained spermatozoa.

In contrast to its effects on the cauda, SK & F 7690 had no effect on the concentration of fructose in the seminal vesicle. At each dose level, the Leydig tissue appeared to be histologically normal but spermatogenesis was always adversely affected. It seems that, in this species, the epididymis and seminal vesicles differ in their sensitivity to SK & F 7690.

INTRODUCTION

The synthetic compound, 17α-methyl-B-nortestosterone (SK & F 7690), bears an overall structural resemblance to testosterone but it is not androgenic, oestrogenic or progestational (Saunders, Holden & Kerwin, 1964). Its anti-androgenic activity has, however, been demonstrated by a number of criteria including inhibition of comb-growth in testosterone-treated chicks (Dorfman, Fajkos & Joska, 1964; Saunders *et al.*, 1964), involution of accessory organs of

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reproduction in intact rats and mice (Saunders *et al.*, 1964; Saunders & Kerwin, 1966), in castrated rats treated with testosterone (Saunders *et al.*, 1964; Mahesh, Zarate, Roper & Greenblatt, 1966; Saunders & Kerwin, 1966) and by the reduction of sebum secretion in ovariectomized rats treated with testosterone (Ebling, 1967).

Hamster spermatozoa isolated in the cauda epididymidis have been found to lose their ability to fertilize 12 days after bilateral gonadectomy, but to retain a high fertilizing capacity in the presence of exogenous testosterone (Lubicz-Nawrocki & Glover, 1973a). The concept that circulating testicular androgens are required to prolong the viability of epididymal spermatozoa has now been tested by using the anti-androgen, SK & F 7690.

MATERIALS AND METHODS

Adult (10-week-old) male and female hamsters were maintained at an environmental temperature of approximately 26° C, and were exposed to 15 hr of light daily. They were allowed free access to Diet 41B (Dixon (Ware) & Sons Ltd) and water.

Treatment of animals

In both test and control animals, each cauda epididymidis was isolated by ligating the distal corpus epididymidis and the ductus deferens at its junction with the cauda epididymidis. Where possible, ligatures were placed beneath blood vessels to minimize damage to the blood supply.

Groups A, B and C consisted of male animals receiving a daily dose of 20, 40 or 60 mg SK & F 7690/100 g body weight, respectively, over a period of 12 days. Group D consisted of animals treated with testosterone (25-mg implant, Organon Laboratories Ltd) and twelve daily injections of 60 mg SK & F 7690/100 g body weight. Animals of Group E were subjected to the same treatment but were also bilaterally gonadectomized (the testes were removed and the ligated epididymides allowed to remain in the scrotum). The control group comprised three animals injected daily for 12 days with oil alone.

The anti-androgen was administered daily as a fine suspension in 0·2 ml arachis oil.

Experimental design

Animals in each group were killed after 12 days and the fertilizing ability of epididymal spermatozoa was tested (see below).

The viability of spermatozoa

Fertilization rate was used as a measure of the fertilizing ability of spermatozoa. Approximately 12×10^6 spermatozoa taken from the cauda epididymidis and suspended in Hanks' solution were injected into each uterine horn of three females in oestrus and eggs were flushed from the uterine tubes 30 to 36 hr after ovulation (Ward, 1946; Strauss, 1956). Whole mounts were prepared (Chang, 1952) and the eggs were examined under the phase contrast microscope and the presence of a headless spermatozoon in the vitellus of a cleaved

egg was taken as the sign of fertilization (Austin, 1956). The number of eggs fertilized, given as a percentage of those which were recovered, expressed the fertilization rate.

The percentage of live (partially stained) spermatozoa (see Lubicz-Nawrocki & Glover, 1973a) was estimated from smears of spermatozoa stained with aqueous nigrosin–eosin, by counting 100 spermatozoa in each of three separate smears. The concentration of spermatozoa injected into the uterine horns was estimated from haemocytometer counts and since the percentage of live spermatozoa in a sample was known from the smears, it was possible to estimate the number of live spermatozoa injected into each female.

Behavioural observations

In addition to examining its effects on the fertilizing capacity of epididymal spermatozoa, an additional series of tests was undertaken to examine whether SK & F 7690 had any effects on libido. For this, treated males were placed together with females in oestrus and the ability to mount females exhibiting lordosis was taken as evidence that libido was unimpaired.

Histological observations

Pieces of testis and cauda epididymidis were fixed for 24 hr in Bouin's fixative, dehydrated, embedded in paraffin wax and sectioned at 7 μm. The sections were stained with haematoxylin and eosin and mounted in DPX.

Estimation of fructose in the seminal vesicles

A further test for the anti-androgenic effects of SK & F 7690 was the estimation of fructose concentration in the seminal vesicles (Mann, 1964), since this is well known to be androgen-dependent. The method was a modification of that of Lindner & Mann (1960) and has been previously described for the hamster by Lubicz-Nawrocki & Glover (1973a).

All data obtained in the experiment were analysed by the Student t test.

RESULTS

Table 1 and Text-fig. 1 show the fertilizing capacity of epididymal spermatozoa is inversely related to the dose of SK & F 7690. At the highest dosage (60 mg/100 g body wt), the mean fertilization rate was reduced to 17·9% of the control level (17·5 versus 97·6%) after 12 days of epididymal ligation. Although a uniform response was obtained with the 60-mg dosage, more variation (as indicated by the large standard error) was observed in fertilizing ability when lower doses were administered (Table 1). Thus, treatment with the 40-mg dose was necessary to produce a significant reduction of the mean fertilization rate ($P<0{\cdot}01$).

However, when testosterone-treated entire animals (Group D) received daily injections of 60 mg SK & F 7690, the epididymal spermatozoa retained a high fertilizing capacity (85·2%$\pm$4·8) whereas the same treatment in bilaterally gonadectomized animals also receiving testosterone replacement (Group E) reduced the mean fertilization rate to 21·6% (Table 1 and Text-fig. 2).

Table 1. The fertilizing capacity of hamster epididymal spermatozoa after treatment with SK & F 7690

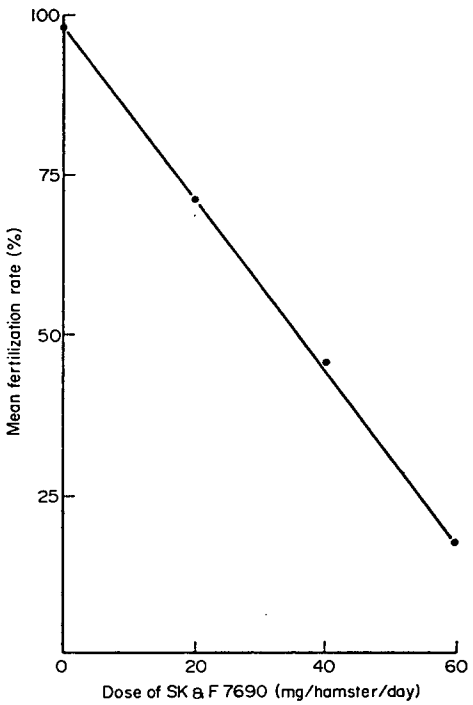
Animal group	*Treatment*	*Mean seminal vesicle fructose (mg/g dry wt ± S.E.)*	*Mean no. of live sperm. × 10^6/ uterine horn ± S.E.*	*Total mean no. of eggs recovered (range)*	*Total mean no. of eggs fertilized*	*Mean fertilization rate in % ± S.E.*
Control	Oil injection	162 ± 6	6·0 ± 0·5	29 (25 to 31)	28·3	97·6 ± 0·9
A	20 mg SK & F 7690	162 ± 2	5·9 ± 0·2	29·2 (22 to 35)	19·8	71·6 ± 13·9
B	40 mg SK & F 7690	160 ± 16	3·3 ± 0·5	29·4 (22 to 34)	11·0	45·9 ± 10·9
C	60 mg SK & F 7690	202 ± 24	4·3 ± 0·7	27·8 (26 to 32)	4·0	17·5 ± 2·0
D	Testosterone + 60 mg SK & F 7690	178 ± 8	3·8 ± 0·1	31 (28 to 33)	25·4	83·2 ± 4·8
E	Castration + testosterone + 60 mg SK & F 7690	188 ± 8	3·9 ± 1·2	28 (23 to 30)	6·0	21·6 ± 3·8

Effect on spermatozoa

Treatment with SK & F 7690 significantly reduced the proportion of live epididymal spermatozoa in some cases (Groups B and D; $P<0{\cdot}01$). There was, however, no correlation between the number of live spermatozoa placed in the female tract and fertilizing capacity (Table 1) so that the poor fertilizing capacity in spermatozoa from the animals in Groups C and E was not due to an increase in the proportion of dead spermatozoa.

Seminal vesicle fructose

Injections of SK & F 7690 had no effect on the mean fructose concentration in the seminal vesicles (Table 1 and Text-fig. 2) in animals from any experimental group.



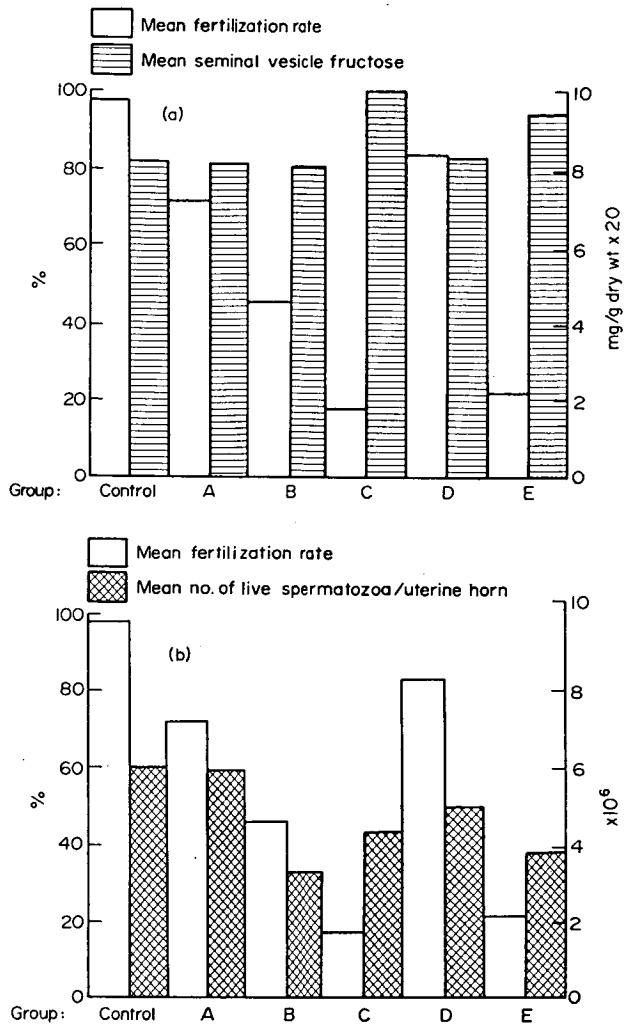
TEXT-FIG. 1. The effect of graded doses of SK & F 7690 on the fertilizing capacity of hamster epididymal spermatozoa (Groups A, B and C).

Histological effects

Testis. In addition to its effect on sperm viability, a 10-mg dose of SK & F 7690 inhibited spermatogenesis at the primary spermatocyte stage (Group A). The severity of the effect was dose-dependent since with a 60-mg dose of SK & F 7690 most of the tubules were aspermic (Pl. 1, Fig. 2). These changes were not prevented by exogenous testosterone (Pl. 1, Fig. 3).

By contrast, the Leydig cells showed no change with any dose of the anti-androgen but appeared to have atrophied when both exogenous androgen and the anti-androgen were present (Group D).

Epididymis. Examination of material from animals in Groups A, B and C revealed that, within 12 days, treatment with anti-androgen resulted in a partial flattening of the principal cells of the tubular epithelium and a pronounced reduction in the number of clear cells which are normally frequently interspersed between the principal cells. A similar change in the distribution of these



TEXT-FIG. 2. (a) The mean fertilizing capacity of hamster epididymal spermatozoa and mean seminal vesicular fructose concentration following various treatments with SK & F 7690 (see text). (b) The mean fertilizing capacity of hamster epididymal spermatozoa and the mean number of live spermatozoa injected *in utero* 12 days after initiation of treatment with SK & F 7690.

cells was previously observed in the hamster 8 to 12 days after bilateral gonadectomy (Lubicz-Nawrocki & Glover, 1973a).

Effect on behaviour

In contrast to its effects on fertilizing capacity, SK & F 7690 did not impair

PLATE 1

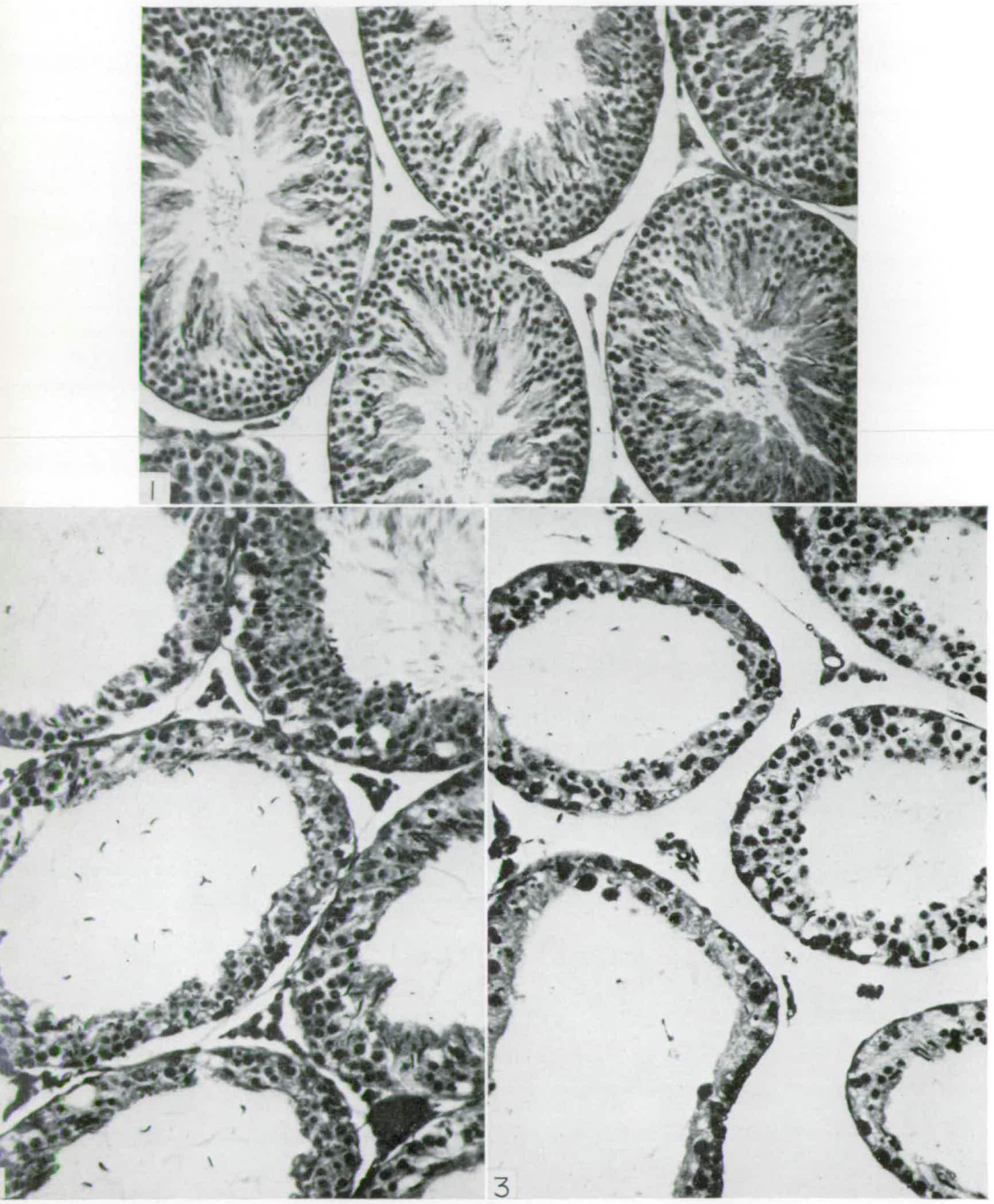


FIG. 1. Section of hamster testis after 12 days of isolation of the cauda epididymidis (control). × 110.

FIG. 2. Hamster testis after 12 daily subcutaneous injections of 60 mg SK & F 7690 (Group C). × 110.

FIG. 3. Hamster testis after 12 daily subcutaneous injections of 60 mg SK & F 7690 following treatment with testosterone (Group D). × 110.

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libido. In each case, the animals retained the ability to mount females that exhibited lordosis.

DISCUSSION

These results show that the anti-androgen SK & F 7690 rapidly destroys the fertilizing capacity of mature epididymal spermatozoa in the golden hamster. Moreover, in gonadectomized animals, twelve daily doses of the steroid at a level of 60 mg/100 g body wt prevent the replacement effect of testosterone. This contrasts with the situation in entire animals with testosterone implants, in which SK & F 7690 appears to be relatively ineffective.

Whether or not the action of SK & F 7690 is mediated directly, say by affecting androgen receptor sites in the epididymis, or indirectly by influencing feed-back mechanisms, is a matter of conjecture and an effect on the spermatozoa themselves, whilst unlikely, cannot be entirely discounted.

The cauda epididymidis and the seminal vesicles are both functionally dependent upon androgen and, since epididymal function was so readily impaired by SK & F 7690, it is perhaps surprising that there was no effect on the seminal vesicles, particularly since this anti-androgen causes regression of all accessory organs in rats and mice (Saunders *et al.*, 1964; Saunders & Kerwin, 1966). It is possible that the anti-androgen is simply unable to interfere with the androgen receptors in the seminal vesicle so that fructose synthesis remains unimpaired. However, Tveter (1971) has shown that SK & F 7690 can reduce the uptake of 5α-dihydrotestosterone (thought to mediate the effects of testosterone) by prostatic cell nuclei, and the cells of the seminal vesicle might be expected to respond similarly. It appears that, in the hamster, there is a variation in the response of different accessory organs to anti-androgen and the epididymis seems to be more sensitive than the seminal vesicles.

Alternatively, like the anti-androgen, cyproterone acetate, SK & F 7690 might compete with androgen feedback on the hypothalamus and increase the secretion of testicular androgens in response to a resulting increase in LH secretion (Neumann, von Berswordt-Wallrabe, Elger, Steinbeck, Hahn & Kramer, 1970). This might then compensate for anti-androgenic activity in the seminal vesicle so that seminal vesicular fructose remains high. This is in line with the work of von Berswordt-Wallrabe & Neumann (1967, 1968) in rats, and that of Voigt, Apostolakis & Klosterhalfen (1968) in man following treatment with cyproterone, and parallels the results of Prasad, Singh & Rajalakshmi (1970), who obtained similar effects in rats with cyproterone acetate. If this were the only explanation of the mechanism of action, however, the response of the epididymis would seem to be paradoxical, so local effects are probably involved.

The changes observed in the tubular epithelium of the isolated cauda epididymidis after treatment with SK & F 7690 closely resemble those which follow androgen withdrawal after castration (Lubicz-Nawrocki & Glover, 1973a) and the almost complete absence of 'clear' cells supports the suggestion of Martan & Risley (1963a, b) that renewal of these cells is androgen-dependent.

In addition to its effects on the cauda epididymidis, SK & F 7690 had an

inhibitory effect on spermatogenesis, even at the lowest doses. This is surprising in view of the compound being generally regarded as a weak anti-androgen and at this stage, the mode of action on the spermatogenic epithelium can only be surmised. Synthesis of FSH might be inhibited, FSH release might be prevented, or both effects might occur. On the other hand, the drug might have a local anti-gonadotrophic action (Mahesh *et al.*, 1966) on the seminiferous tubules themselves. In this connection, it is interesting that PMSG maintains normal spermatogenesis in hamsters following hypophysectomy (R. P. Millar, personal communication) whereas HCG or testosterone alone will not (Lubicz-Nawrocki & Glover, 1973a, b).

An anti-fertility action of orally administered SK & F 7690 after 35 to 49 days has been reported in mice (Saunders, Tomaszewski, Pauls & Zuccarello, 1969), the suggestion being that decreased libido or failure to copulate properly were the underlying causes. In the present work, SK & F 7690 did not affect libido and although the effects of long-term treatment were not examined, initial loss of fertility was due rather to an effect on the spermatozoa.

The present findings show that anti-androgen impairs viability in mature epididymal spermatozoa and in this regard, SK & F 7690 simulates the effects of bilateral gonadectomy. The effects would appear to be due to a competitive inhibition of androgen upon which the cells lining the epididymis are known to be so dependent. Further investigation is needed to determine the precise mechanism of action of both androgen and anti-androgen on epididymal cells, for at the time of writing, there is no clear information on these subjects.

ACKNOWLEDGMENTS

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THE EFFECTS OF CASTRATION ON THE COMPOSITION OF RABBIT EPIDIDYMAL PLASMA

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Summary. The composition of luminal plasma from the cauda epididymidis of rabbits has been investigated after prolonged retention of spermatozoa in the cauda epididymidis of entire animals, and of castrated animals both with and without hormone replacement. The morphology of the contained spermatozoa and the epididymal cells has also been examined.

In the presence of androgen, spermatozoa survived for 4 to 5 weeks before degenerative changes became apparent and the composition of the epididymal plasma and the histological characteristics of the lining cells were not seriously affected during this period. The only change in the plasma which could be due to sperm death was an increase in the lactic dehydrogenase (LDH) activity. After androgen withdrawal, spermatozoa degenerated rapidly and there were considerable changes in the composition of the luminal plasma. There was a rapid increase in the level of sodium ions and pH, and a decrease in the concentrations of glycerylphosphorylcholine (GPC) and protein. The activity of acid phosphatase and LDH also decreased. Initially, the concentration of inorganic phosphate and potassium increased but this was soon followed by a decrease. Five weeks after castration, all the spermatozoa disappeared from the epididymis and the epididymal cells had regressed. If testosterone replacement was given at this stage, the level of sodium ions in the plasma decreased and the concentration of GPC increased. The epididymal cells were also restored to their normal histological appearance.

These results provide evidence that, in the presence of androgen, the epididymal cells in the cauda epididymidis actively maintain a constant *milieu* in the lumen of the duct by their capacity for absorption and secretion. If androgen is withdrawn, these functions cease and considerable changes take place in the lumen.

INTRODUCTION

The prolonged viability of spermatozoa in the cauda epididymidis of mammals

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has been well documented (Hammond & Asdell, 1926; Snell, 1933; White, 1933; Paŭfler & Foote, 1968; Tesh & Glover, 1969). It is also known that the epididymal epithelium is dependent on circulating androgen (see Mann, 1964), and that if androgen is withdrawn by means of castration, then sperm survival in the cauda epididymidis is drastically curtailed (Mann, 1964; Lubicz-Nawrocki & Glover, 1970). However, there is no information in the literature on the chemical nature of the *milieu* of spermatozoa in the cauda epididymidis during their prolonged retention therein or after androgen withdrawal. It has been shown that the fluid or 'plasma' surrounding spermatozoa in the lumen of the cauda epididymidis is very different from that of other body fluids (Scott, Wales, Wallace & White, 1963; Crabo & Gustafsson, 1964; Mann, 1964; Crabo, 1965; Wales, Wallace & White, 1966; Jones, 1973) and it may be that this is related to the prolonged survival of spermatozoa in this part of the duct.

In the present study, the composition of the luminal plasma from the cauda epididymidis of rabbits has been investigated under different hormonal conditions, and an attempt has been made to relate the composition of plasma to the morphology of epididymal spermatozoa and the function of the epididymal cells.

MATERIALS AND METHODS

A total of sixty-nine adult male rabbits of mixed strain was used in four experiments. Animals were caged individually and maintained on a diet of SGl pellets (Nutrients Ltd, Liverpool) and water. One week before surgery, all rabbits were allowed to ejaculate once and only those with good quality semen were used.

Twelve adult males were anaesthetized with an intravenous injection of sodium pentabarbitone (Nembutal, Abbott Laboratories) and were bilaterally castrated through a scrotal incision. Two ligatures, one on the distal part of the corpus epididymidis and another on the ductus deferens, were used to isolate the cauda epididymidis and to prevent the in- or outflow of spermatozoa. Care was taken to avoid undue disturbance to the blood supply when applying the ligatures. The epididymides were returned to the scrotum and the incisions were closed with interrupted nylon sutures and 'Octaflex' antiseptic plastic dressing (Ward Blenkinsop & Co., London). Recovery was uneventful and these animals are referred to hereafter as the 'castrate group'.

A further eighteen rabbits were castrated and the epididymides were ligated in a similar fashion, but they were given a subcutaneous implant (50 mg) of testosterone propionate (Organon Laboratories, London) at the time of castration. These animals constituted the 'hormone replacement group'. In an additional eighteen males, the 'ligated control group', the testes remained intact and the cauda epididymidis was isolated with ligatures.

In order to investigate the effects of testosterone replacement after a prolonged period of castration, twenty-one males were bilaterally castrated and only received an implant of testosterone propionate 5 weeks later. These males were referred to as the 'delayed replacement group'.

The contents of the cauda epididymidis were collected from three animals

per week in each treatment group, using the cannulation procedure of Jones & Glover (1973). The experiment was terminated after 8 weeks in the 'ligated control' and 'hormone replacement' groups, after 7 weeks in the 'delayed replacement group', and after 5 weeks in the 'castrate group'. The epididymal contents from each animal were treated separately, and after centrifugation at 12,000 *g* for 5 min, the spermatocrit (packed cell volume) and 'volume of plasma collected' per rabbit were recorded. The plasma was stored under mineral oil at −20°C, and was then analysed for sodium and potassium ions, total protein, inorganic phosphate, glycerylphosphorylcholine (GPC), acid phosphatase, alkaline phosphatase, lactic dehydrogenase (LDH), α-mannosidase, β-*N*-acetylglucosaminidase, osmotic pressure and pH (see Jones & Glover, 1973).

Before centrifugation, samples of epididymal contents were mixed with a drop of aqueous nigrosin–eosin for 5 min (Campbell, Dott & Glover, 1956) and smeared on clean glass slides. Percentages of stained ('dead') and decapitate spermatozoa were estimated from counts of 300 spermatozoa in duplicate smears.

The epididymides were removed after collection of the epididymal contents, fixed in Bouin's fluid, and processed by routine histological methods. Sections of the cauda epididymidis were cut at 6 μm, stained with Erlich's haematoxylin and eosin, and mounted with DPX mounting medium. Measurements of the diameter of the lumen and of the height of the epithelial cells were made with a micrometer eye-piece at magnifications of ×40 and ×400.

Peripheral blood was collected from the marginal ear vein and testosterone levels were estimated by means of radioimmunoassay.

All these results were assessed for significance using the Student *t* test.

RESULTS

The results for the first three experiments are shown in Tables 1 to 4. They show that after castration, spermatozoa in the cauda epididymidis died and disintegrated so that by the 5th week, they had disappeared completely and the spermatocrit was zero (Table 1 and see Pl. 1, Fig. 2). In the 'ligated control' and 'hormone replacement' groups, however, significant degeneration within the sperm population was not apparent until 3 to 5 weeks had elapsed. When hormone was present, the spermatozoa did not disappear and during this period, any decrease in spermatocrit could be adequately accounted for by an increase in the volume of plasma collected. There was, in fact, a significant negative correlation between the spermatocrit and the volume of plasma collected (castrate group, $r = 0{\cdot}862$, $P<0{\cdot}01$; hormone replacement group, $r = 0{\cdot}613$, $P<0{\cdot}01$).

In the 'castrate group', an increase in the volume of plasma collected was accompanied by considerable changes in its composition. The most noticeable of these was a rapid increase in levels of sodium and potassium ions and inorganic phosphate, and a decrease in the concentrations of GPC and total protein and in the activity of acid phosphatase (Tables 2 and 3). It was noticeable that on the 1st week after castration, there was a transient increase in the

Table 1. Morphological characteristics of spermatozoa, spermatocrit and volume of plasma collected from the cauda epididymidis of the rabbit

Experimental group	*Weeks after operation*	*% stained spermatozoa*	*% decapitate spermatozoa*	*% spermatocrit*	*Volume of plasma collected (µl)*
Normal (intact)		7·3± 2·7	0·6± 0·4	20·4±2·0	75·2±28·8
Ligated control group	1	11·7± 2·1	3·5± 1·2	21·7±2·1	112·2±25·4
	2	12·8± 1·8	5·1± 0·7	23·4±3·5	158·7±44·8
	3	13·9± 0·9	9·7± 4·1	24·9±4·5	221·2±31·5
	5	39·9±12·8	16·4± 6·5	16·1±3·8	212·8±22·3
	6	99·2± 1·7	65·8± 6·2	13·3±1·2	368·6±65·1
	8	96·3± 1·2	39·9± 8·3	8·5±1·9	360·1±71·2
Castrated group	1	22·3± 2·1	4·3± 2·1	*29·9±2·2	151·4±21·1
	2	**89·0± 4·7	**28·7± 3·7	20·3±0·8	223·6±65·4
	3	**100·0± 0·0	**79·5± 6·4	**5·6±1·3	327·5±84·1
	5	—	—	**0·0	365·1±30·5
Hormone replacement group	1	9·3± 5·2	2·8± 6·4	29·7±5·9	87·2±19·1
	2	12·8± 3·3	3·9± 2·1	23·7±5·3	142·0±48·3
	3	17·9± 4·8	6·7± 7·3	14·7±5·3	222·7±51·9
	5	92·8± 2·7	43·1±19·5	9·7±2·1	290·6±64·7
	6	96·1± 0·3	57·3±12·1	11·6±1·3	328·1±29·1
	8	97·8± 9·4	39·6±10·8	5·8±0·9	335·0±28·3

Values for normal animals are taken from Jones & Glover (1973). Each value is the mean ±S.E. for three animals.

* Significantly different from ligated controls ($P<0·05$).
** Significantly different from ligated controls ($P<0·01$).

Table 2. Chemical composition of epididymal plasma from the cauda epididymidis of the rabbit

Experimental group	*Weeks after operation*	*Na^+ (mequiv./litre)*	*K^+ (mequiv./litre)*	*GPC (mg/100 ml)*	*Inorganic phosphate (mg P/100 ml)*
Normal (intact)		20·7± 2·3	24·8± 1·2	766·6± 41·6	12·42±1·0
Ligated control group	1	16·9± 0·6	20·4± 0·8	614·0± 30·2	14·2 ±1·3
	2	14·4± 0·8	19·9± 1·2	687·3± 24·2	16·7 ±1·1
	3	13·2± 0·7	15·4± 1·0	670·6± 16·5	20·3 ±1·2
	5	17·1± 0·2	20·1± 9·1	705·3± 35·6	22·6 ±2·9
	6	18·4± 0·4	16·9± 2·7	729·6± 16·4	28·5 ±4·1
	8	14·3± 0·3	16·4± 3·4	694·1± 98·1	22·6 ±2·3
Castrated group	1	27·6± 4·8	25·2± 3·1	**1155·9± 67·9	*33·2 ±2·1
	2	**54·6± 4·2	**35·9± 2·4	815·2± 60·4	**53·9 ±3·2
	3	**80·5±30·3	*47·9±11·0	308·4±105·0	14·1 ±1·7
	5	**86·5± 8·4	22·9± 5·7	**18·2± 8·4	**3·2 ±0·4
Hormone replacement group	1	17·5± 0·3	22·4± 1·3	706·1± 29·0	12·7 ±0·4
	2	14·1± 0·6	20·3± 1·1	791·4± 27·3	15·8 ±0·9
	3	11·3± 1·1	24·6± 0·4	815·4± 98·4	20·2 ±2·3
	5	14·5± 1·2	17·2± 0·7	774·6± 8·3	24·7 ±2·3
	6	13·6± 0·8	16·5± 1·8	574·1± 31·7	27·8 ±3·1
	8	12·8± 0·4	18·2± 1·4	830·4± 35·4	26·6 ±1·3

Each value is the mean ±S.E. for three animals.

* Significantly different from ligated controls, $P<0·05$.
** Significantly different from ligated controls, $P<0·01$.

Table 3. Chemical composition of epididymal plasma from the cauda epididymidis of the rabbit

Experimental group	*Weeks after operation*	*Total protein (g/100 ml)*	*Osmotic pressure (ΔT_f°C)*	*Acid phosphatase (i.u.)*	*LDH (i.u.)*
Normal (intact)		4·03±0·23	0·580±0·035	106 ± 41·3	648± 91
Ligated control group	1	4·80±0·59	0·604±0·015	239 ± 11·4	718± 134
	2	5·18±0·33	0·580±0·021	357 ± 46·7	2200± 546
	3	4·83±0·26	0·554±0·028	402 ±100·9	3442±1121
	5	6·35±0·26	0·704±0·021	336 ± 11·4	5835±3421
	6	6·35±1·15	0·714±0·110	452 ± 32·8	11,565±1340
	8	6·23±1·17	0·616±0·013	433 ±210·1	6974±2078
Castrated group	1	6·89±1·17	0·582±0·071	250 ±810	2727± 584
	2	6·23±1·41	0·617±0·094	261 ± 21·4	721± 210
	3	3·21±1·36	0·426±0·036	201 ± 64·2	2104±1111
	5	**0·64±0·26	0·590±9·101	**27·6± 10·3	**456± 104
Hormone replacement group	1	5·17±0·64	0·545±0·019	390 ± 15·7	2653± 103
	2	5·68±0·71	0·621±0·028	423 ± 71·2	1271± 234
	3	5·54±0·68	0·707±0·036	417 ± 94·8	2480±1225
	5	6·4 ±0·35	0·614±0·041	389 ± 24·7	1515± 138
	6	6·08±0·33	0·689±0·040	**755 ± 74·1	6916±2890
	8	6·13±1·08	0·710±0·060	413 ±101·2	2827±1009

Each value is the mean ±S.E. for three animals. i.u. = International Units (μmol substrate hydrolysed/min/litre of plasma at 37° C).

** Significantly different from ligated controls, $P<0·01$.

concentrations of GPC and protein and also in the spermatocrit reading, but this was reversed by the 5th week when low readings were obtained in each case. The activity of LDH also decreased to low levels after 5 weeks.

In the 'ligated control' and 'hormone replacement' groups, the composition of the epididymal plasma did not alter drastically from that in normal (unligated) animals (see Jones & Glover, 1973). The levels of sodium, potassium

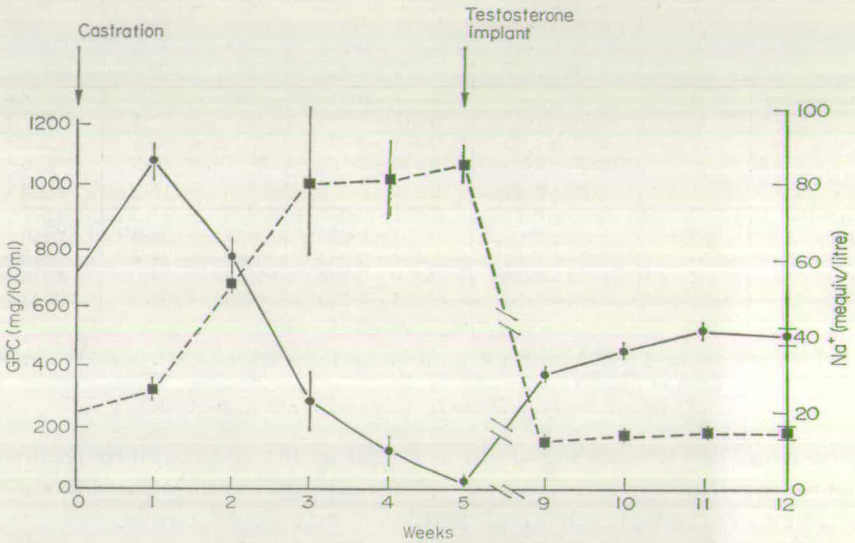
Table 4. Mean lumen diameter and epithelial cell height of the cauda epididymidis of the rabbit during castration for 5 weeks followed by hormone replacement

Weeks after castration	*Lumen diameter (μm)*	*Epithelial cell height (μm)*
Normal animal	654·0±26·4	16·2±0·8
1	609·7±33·8	16·0±0·9
2	574·1±41·7	10·8±0·6
3	398·9±29·2	6·1±0·4
5	374·4±54·9	5·1±0·1
Testosterone implant		
6	90·3± 9·8	25·8±1·2
7	364·7±18·9	14·8±0·9
8	358·1±36·7	16·9±1·7
9	360·5±18·7	16·3±1·0
10	422·4±21·7	19·5±1·5
11	418·7±31·7	20·8±2·7
12	454·9±33·2	15·7±0·9

Values are the mean ±S.E. for three animals. Measurements were made on seven tubules per section for five sections of the cauda epididymidis of each animal.

and GPC remained within normal limits also, although there was a slight increase in the concentrations of protein and inorganic phosphate, and in acid phosphatase activity. The only change was an increase in the level of LDH activity during the 5th and 6th weeks, to be followed by a decrease during the 8th week (Table 3). This was in distinct contrast to the situation after castration.

Of the other enzymes studied, namely alkaline phosphatase, β-*N*-acetyl-glucosaminidase and α-mannosidase, there was a similar pattern of change to



TEXT-FIG. 1. The concentrations of GPC (●) and Na^+ ions (■) in rabbit epididymal plasma after castration and after castration followed by a testosterone implant on the 5th week (see text). Each value is the mean ±S.E. for three animals.

that observed for acid phosphatase so that after 5 weeks of castration, their activity in the plasma had decreased to less than 20% of that in the 'ligated controls'.

The osmotic pressure measurements (Table 3) deserve further mention, however, since after castration, the osmotic pressure of the epididymal plasma was not significantly less than that in the plasma of ligated controls where it always remained hypertonic to blood (blood plasma, $\Delta T_f = 0{\cdot}572°$ C). By

EXPLANATION OF PLATE 1

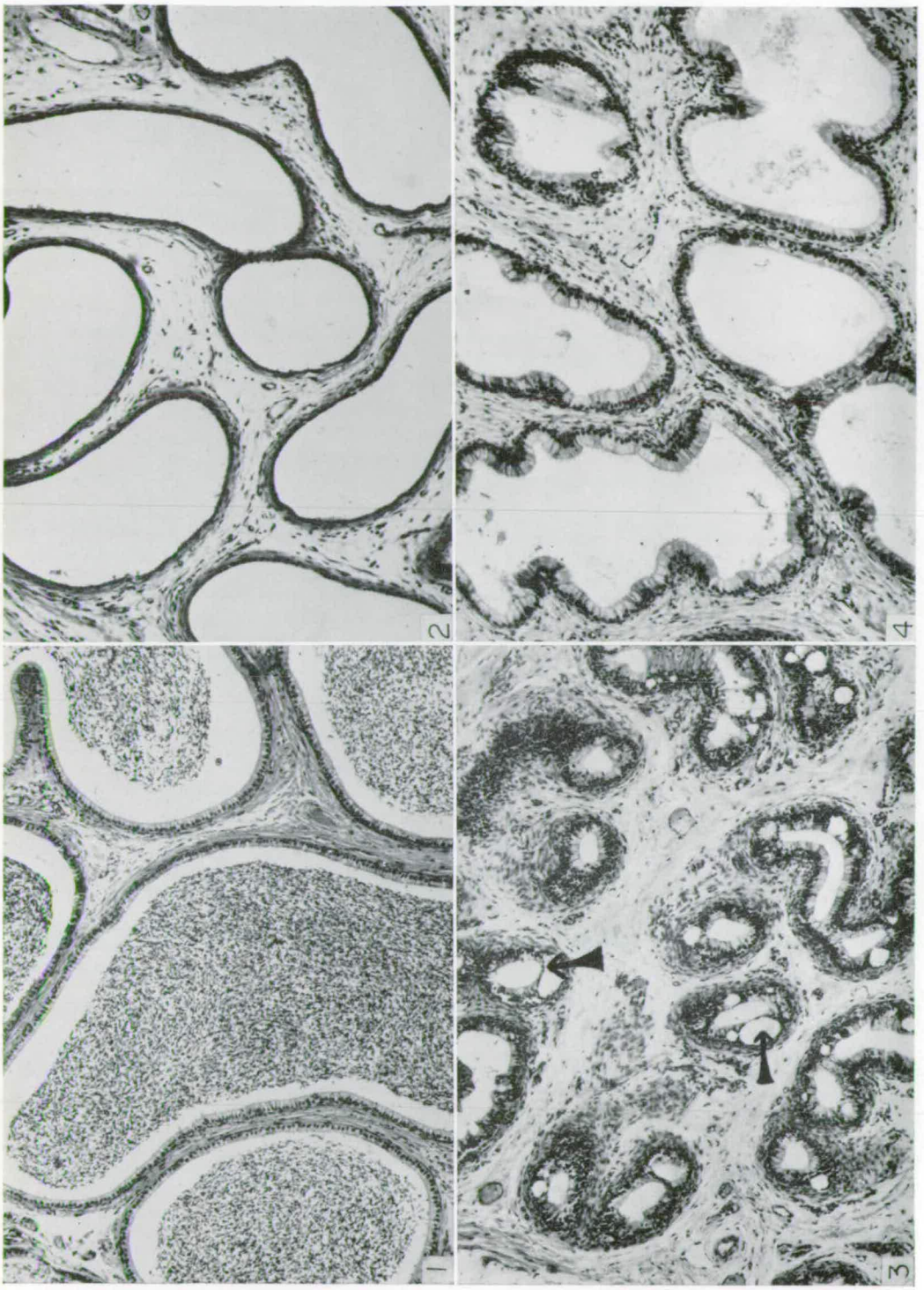
FIG. 1. Histological section of the cauda epididymidis of a control rabbit 5 weeks after ligation (see text). The lumen is packed with spermatozoa. Haematoxylin and eosin. ×70.

FIG. 2. Histological section of the cauda epididymidis of a rabbit 5 weeks after castration (see text). Note the wide lumen, absence of spermatozoa, and the total regression of the epididymal epithelium. Haematoxylin and eosin. ×70.

FIG. 3. Histological section of the cauda epididymidis of a rabbit 5 weeks after castration and 1 week after being given a testosterone implant. Note the small lumen diameter. Folds in the epithelium appear as 'crypts' (arrowed). Haematoxylin and eosin. ×70.

FIG. 4. Same as Fig. 3, but 5 weeks after receiving the testosterone implant. Note the reappearance of the wide lumen and the restoration of the epididymal epithelium (see Table 4). Haematoxylin and eosin. ×70.

PLATE 1



(*Facing p.* 410)

contrast, the pH of the plasma increased rapidly after castration and reached mean values on the 1st, 2nd, 3rd and 5th weeks of 6·8, 7·2, 7·7 and 8·05, respectively. In the 'ligated control' and 'hormone replacement' groups, the pH of the plasma remained below 7·0 for the duration of the experiment.

After castration, the epididymal epithelium began to regress and by the 5th week, the cell height was greatly reduced and the stereocilia had disappeared completely (see Table 4 and Pl. 1, Fig. 2). The flattened appearance of the epithelium in these circumstances was due to the accumulation of 'fluid' (see earlier) which was prevented from leaving the epididymis by the ligature on the ductus deferens. If this ligature was omitted, the contents passed freely into the urethra and, in castrated animals, the cauda epididymidis was completely empty by the 4th week. In the 'ligated control' and 'hormone replacement' groups, the epididymal epithelium retained its normal histological appearance.

The results of delaying hormone replacement until 5 weeks after castration are shown in Text-fig. 1 and Pl. 1, Figs 3 and 4. One week after giving the testosterone implant, it was impossible to collect any 'fluid' from the epididymis. The lumen of the tubules had collapsed completely (see Table 4 and Pl. 1, Fig. 3) and it was not until the 4th week that small volumes could be collected again. The diameter of the lumen gradually increased during this time as did the epithelial cell height (Table 4 and Pl. 1, Fig. 4). At these times, when sperm-free 'plasma' could be collected, it was found that the levels of sodium ions had declined to below normal levels while the concentration of GPC had increased (Text-fig. 1). The GPC concentration, however, never reached normal levels even after 7 weeks of hormone replacement when the pH of the plasma had also returned to values below 7·0.

These findings are of added interest when related to the levels of testosterone in the blood. Only two animals were used in this context but it was found that there was a rapid decrease in blood testosterone from over 500 ng/100 ml 30 min before castration, to less than 50 ng/100 ml 2 hr after castration. Twenty-four hours later, the testosterone concentration had fallen to less than 10 ng/100 ml. Within 3 days of giving an implant of testosterone propionate to 35-day castrates, however, the concentration of circulating testosterone increased to over 300 ng/100 ml.

DISCUSSION

This work has shown that the chemical composition of epididymal plasma in the cauda epididymidis is profoundly influenced by circulating androgen.

Isolation of this region by means of ligatures must, in part, create unphysiological conditions but disturbance of the blood supply is not apparent and although the lymphatics might be affected, the composition of the plasma does not appear to alter significantly (see Jones & Glover, 1973). The use of ligatures can be justified on the grounds that any changes in the plasma must be due to altered function in the cauda epididymidis itself, and not to changes in higher regions of the duct.

Nevertheless, it is reasonable to assume that the composition of the plasma is

the result of two interacting sources, the spermatozoa and the epididymal cells. If this is true, the situation might be represented as:

spermatozoa ⇋ epididymal plasma ⇋ epididymis

Changes in the composition of the plasma are thus fairly difficult to interpret, especially if both spermatozoa and epididymal cells are adversely affected. After castration, for example, rapid degeneration of spermatozoa coincides with the regression of epididymal cells. A corresponding increase in the levels of sodium and potassium ions and in the pH of the plasma strongly suggests a reduced absorptive capacity by the epididymis, although degenerating spermatozoa represent another potential source of inorganic ions (Quinn, White & Wirrick, 1966). Similarly, it is difficult to be certain of the origin of high levels of inorganic phosphate 2 weeks after castration. Spermatozoa contain endogenous reserves of phospholipids (Hartree & Mann, 1959, 1961) and nucleotides (Newton & Rothschild, 1961; Brooks, 1970) and it is conceivable that after castration, there is a breakdown of these reserves with a release of free phosphate into the lumen. It is also possible that phosphorus-containing compounds in the plasma itself may be degraded, especially in view of the high levels of phosphatases in the plasma at this time. A third possibility is the direct release of phosphate from the epididymal cells into the lumen. Whatever the origin of phosphate, its interrelationships in the epididymis warrant further investigation.

The only parameter which is not significantly affected by castration is the osmotic pressure. This is maintained within normal levels in spite of a considerable increase in the ionic content of the plasma. A decrease in the concentration of other osmotically active substances in the plasma, therefore, seems likely.

In contrast to the above substances, a decreased concentration of GPC after castration can only be attributed to a reduction in the secretory activity of the epididymal cells. It is well known that GPC is actively synthesized by the epididymis (Dawson, Mann & White, 1957; Scott *et al.*, 1963; Mann, 1964; Wallace, Wales & White, 1966) and that it is not metabolized directly by spermatozoa (White, Wallace, Wales & Scott, 1961). Its occurrence in the epididymis is dependent on the presence of circulating androgen (Dawson & Rowlands, 1959). Thus, in the 'ligated control' and 'hormone replacement' groups, normal secretory activity by the cells of the epididymis would seem to be retained, since the concentration of GPC in the plasma was within normal limits. These concentrations were maintained throughout the experimental periods in spite of spermatozoa degenerating and probably releasing intracellular constituents into the plasma after 4 or 5 weeks. An increase in the level of LDH activity in the plasma indicates that leakage of sperm contents occurred about this time (see Jones & Glover, 1973). If other intracellular constituents such as potassium ions were released, however, their concentration never reached detectable proportions. Even LDH levels declined after about 8 weeks.

It seems, therefore, that in the presence of circulating androgen, the epithelial cells lining the cauda epididymidis serve to maintain a relatively stable luminal environment by virtue of their capacity for absorption and secretion. Further evidence of a potentiality for active secretion is provided by a progressive

increase in the volume of the luminal fluid following isolation of the cauda epididymidis and, since the normal composition of the plasma was maintained over a period of 8 weeks, the increase would not seem to be due to the passive entry of fluid into the lumen. This does not apply to castrated animals, however, in which there is considerable change in the composition of the plasma and where secretion is probably inhibited.

The secretory and absorptive activity of the cells lining the cauda epididymidis seem to differ in their sensitivity to androgen. During the 1st week of castration, secretion appears to be halted but absorption seems to proceed. This should have a concentrating effect and, indeed, the spermatocrit and the concentrations of GPC and protein in the plasma increase during this time. The situation is only rectified later when absorptive activity is, presumably, also adversely affected by the lack of androgen. After a prolonged period of castration followed by testosterone replacement, absorptive activity is restored first so that superfluous fluid is quickly removed and renewed secretion only begins afterwards. This restoration of normal function is characterized by a decrease in the level of sodium ions and an increase in GPC. The GPC never quite reaches normal levels, however, which indicates that normally a proportion of the GPC in the lumen of the cauda epididymidis originates from higher up the duct, bearing in mind that in these experiments the cauda epididymidis was isolated by ligation. It may, of course, also reflect the lower potential of cells in this area for synthesizing GPC (Scott *et al.*, 1963; Wallace *et al.*, 1966).

Overall, therefore, the evidence presented here strongly suggests that in the presence of circulating androgen, the cells lining the cauda epididymidis actively maintain a stable *milieu* in the lumen of the duct. Any disturbance of this *milieu*, such as might result from the degeneration of some of the spermatozoa, seems to be compensated for by the normal secretory and absorptive activity of the epithelium. An important next step would be to determine the exact nature of the secretory and absorptive activity of the epididymal cells and the precise contribution of degenerating spermatozoa to the plasma following castration.

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THE COLLECTION AND COMPOSITION OF EPIDIDYMAL PLASMA FROM THE CAUDA EPIDIDYMIDIS OF THE RABBIT

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Summary. A technique is described for collection of the epididymal contents from the cauda epididymidis of anaesthetized rabbits. The technique avoids undue contamination of the epididymal contents with blood or tissue fluid and overcomes the difficulty of *post-mortem* change.

The effects of high-speed centrifugation and cold shock on the composition of epididymal plasma have also been investigated, and it has been shown that lactic dehydrogenase and glutamic–oxaloacetic transaminase are largely intracellular constituents, whilst sodium, potassium, acid phosphatase, alkaline phosphatase and β-*N*-acetylglucosaminidase are extracellular. Finally, some results are presented on the overall composition of epididymal plasma in the rabbit.

INTRODUCTION

The composition of the fluid or 'plasma' that suspends spermatozoa as they are transported through the epididymis has been investigated on numerous occasions (Scott, Wales, Wallace & White, 1963; Crabo & Gustafsson, 1964; Mann, 1964; Crabo, 1965; Wales, Wallace & White, 1966), but progress in this field has been hampered largely by the difficulties of collecting sufficient quantities of the plasma for analysis. Mostly, slaughterhouse material has been used and the luminal contents have been collected by direct puncture of the epididymal tubules (see Crabo, 1965). This method is open to considerable error as a result of contamination with blood or tissue fluid, and the collection procedure itself may take several hours. The precautions taken by various workers to overcome these problems have varied considerably and this probably accounts for many discrepancies that are reported in the literature.

In addition, the separation of spermatozoa from the plasma is a problem which has not been investigated adequately. It has often been stressed that procedures that injure spermatozoa, such as high-speed centrifugation or cold shock, cause leakage of their intracellular proteins and coenzymes into the supernatant plasma (Mann & Lutwak-Mann, 1955; Mann, 1964; Graham & Pace, 1967; Pace & Graham, 1970). Since centrifugation is commonly used

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for separating epididymal plasma from spermatozoa, some of the constituents of the plasma might have their origin in the spermatozoa and might not be truly extracellular.

The aims of the present work, therefore, were to develop techniques for the collection of epididymal plasma from the cauda epididymidis of anaesthetized rabbits, and to investigate the chemical composition of the plasma so obtained.

MATERIALS AND METHODS

The terminology of Crabo & Gustafsson (1964) is used throughout, so that the term 'epididymal content' refers to both the fluid and spermatozoa in any one region of the epididymis, and 'epididymal plasma' refers only to the supernatant after centrifugation of the epididymal content.

Collection of epididymal contents from the cauda epididymidis

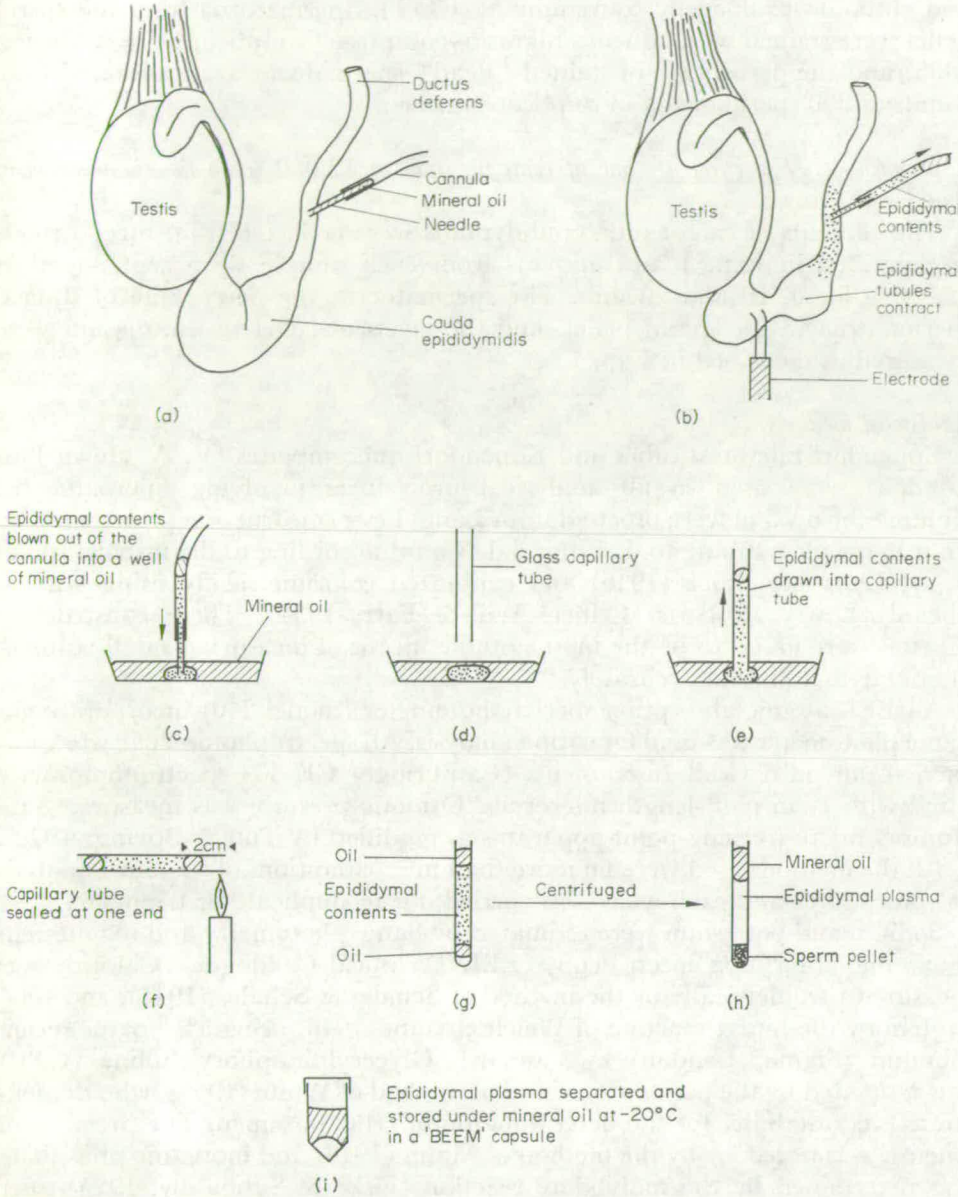
Adult male rabbits were anaesthetized with an intravenous injection of sodium pentabarbitone (Nembutal, Abbott Laboratories), and the testis, epididymis and ductus deferens were exposed through a scrotal incision and placed on a piece of clean gauze. Care was taken not to interfere with the blood supply. The ductus deferens was cannulated close to the cauda epididymidis with a polythene cannula (o.d. 1 mm, i.d. 0·5 mm) partially filled with mineral oil (Nujol, Boots Ltd) and tipped with a 25-gauge needle (Text-fig. 1a). This enabled the cannula to be inserted directly into the lumen of the duct with a minimum of trauma and the mineral oil prevented any blood or tissue fluid from being drawn up the cannula by capillary action. A small metal electrode delivering 0·5 to 1·0 V was used to stimulate contraction of the smooth muscle in the wall of the epididymal tubules and this pumped the epididymal contents out of the more proximal regions of the cauda epididymidis into the ductus deferens where it was sucked up into the cannula (Text-fig. 1b). In this way, volumes of between 25 and 75 μl per epididymis could be collected without difficulty.

The epididymal content was transferred under mineral oil to clean glass capillary tubes (o.d. 1·4 mm, i.d. 1·0 mm, Hawksley Ltd) which were heat-sealed at one end in a hot flame to localize the area of heating (Text-fig. 1 c to f). The epididymal content, trapped between two columns of mineral oil (Text-fig. 1 g to h) was centrifuged in a microhaematocrit centrifuge (Hawksley Ltd) fitted with a Variac transformer. After centrifugation, the 'spermatocrit' or packed cell volume, expressed as a percentage, and the volume of plasma collected were recorded. The plasma was then separated and stored in polythene 'BEEM' capsules (TAAB Laboratories) under mineral oil at −20° C (Text-fig. 1 i).

The following experiments were carried out in order to determine the conditions of centrifugation under which there was maximal separation of spermatozoa together with a minimal effect on the composition of the supernatant plasma.

Experiment 1. *The effect of centrifugation on the composition of the epididymal plasma*

The contents of each cauda epididymidis were collected from three rabbits



TEXT-FIG. 1. Flow diagram of the technique used for collection of the epididymal plasma from the cauda epididymidis of rabbits (see text).

and samples from each animal were subjected to three different treatments. Sample 1 was centrifuged at 4000 *g* for 5 min, Sample 2 at 12,000 *g* for 5 min and Sample 3 was subjected to cold shock by plunging onto ice at −20° C for 10 min before centrifuging at 12,000 *g* for 5 min. The spermatocrit was measured and the plasma analysed for sodium, potassium, alkaline phosphatase, acid phosphatase, *β*-*N*-acetylglucosaminidase, lactic dehydrogenase (LDH)

and glutamic–oxaloacetic transaminase (GOT). Spermatozoa from the sperm pellet were stained with aqueous nigrosin–eosin (see Campbell, Dott & Glover, 1956) and the percentage of stained ('dead') spermatozoa was estimated from counts of 300 spermatozoa in duplicate smears.

Experiment 2. *The effect of time of centrifugation at* 12,000 **g** *on the composition of epididymal plasma*

The contents of each cauda epididymidis were collected from three rabbits as described in Exp. 1 and aliquots from each sample were centrifuged at 12,000 *g* for 5, 10 and 20 min. The spermatocrit, the percentage of stained spermatozoa in the sperm pellet, and the levels of LDH in the plasma were measured as described in Exp. 1.

Analytical methods

Eppendorf microtest-tubes and Eppendorf micropipettes (V. A. Howe Ltd, London) were used in all analytical procedures involving microanalysis. Volumes below 5 μl were pipetted from Lang–Levy construction pipettes pulled from Pyrex glass tubing (o.d. 5 mm, i.d. 3 mm) according to the instructions of Bessy, Lowry & Brock (1946) and calibrated colorimetrically using *p*-nitrophenol (Lowry, Roberts, Leiner, Wu & Farr, 1954). These construction pipettes were found to be the most suitable means of measuring small volumes of epididymal plasma accurately.

An EEL atomic absorption spectrophotometer (model 240) incorporating a flame photometer was used for cation analysis. All spectrophotometric work was carried out on a Cecil Instruments (Cambridge) CE 303 spectrophotometer fitted with 1-cm path-length microcells. Osmotic pressure was measured on a Ramsay micro-freezing-point apparatus as modified by Tuft & Böving (1970).

All the methods used were no more than microadaptions of standard analytical procedures and each assay was carried out in duplicate or triplicate.

Sodium and potassium were estimated by flame photometry and magnesium by atomic absorption spectroscopy (EEL Technical Catalogue). Chloride was measured titrometrically by the method of Schales & Schales (1941), and total protein by the Biuret reaction of Weichselbaum (1946) using 5% bovine serum albumin (Sigma, London) as standard. Glycerylphosphorylcholine (GPC) was estimated by the periodate oxidation method of White (1959), who demonstrated its suitability for the determination of GPC in semen. The presence of fructose was tested for by the method of Mann (1946) and inorganic phosphate was determined by the molybdate reaction (Fiske & Subbarow, 1925) after deproteinization with ice-cold 10% (w/v) trichloroacetic acid (TCA). Acid phosphatase and alkaline phosphatase were measured colorimetrically, using *p*-nitrophenylphosphate as substrate (Boehringer test kits) and LDH and GOT were determined spectrophotometrically at 340 mm (Boehringer test kits). α-Mannosidose and β-*N*-acetylglucosaminidase were estimated by the method of Conchie, Findlay & Levvy (1959), using the corresponding *p*-nitrophenylglycoside as substrate (Sigma). Osmotic pressure was determined as freezing-point depression (Tuft & Böving, 1970) and pH was estimated by pipetting 1-μl aliquots of plasma directly onto Universal pH paper (Microessentials

Laboratories, New York). The colour was compared to that developed by buffers in the appropriate pH range and treated in a similar fashion.

RESULTS

The results of Exp. 1 (Table 1) show that increasing the force of centrifugation from 4000 *g* to 12,000 *g* gives rise to a decrease in the spermatocrit from 40·9% to less than 20·0% (difference highly significant, $P<0·001$). At the same time there was an increase in the incidence of stained spermatozoa in the sperm pellet; the difference between values after centrifugation at 4000 *g* and 12,000 *g* was not significant ($P<0·05$), but both values were significantly different from spermatozoa which had been subjected to cold shock and centrifuged at 12,000 *g* ($P<0·001$).

Table 1. The effect of the force of centrifugation and cold shock on the incidence of stained spermatozoa in the sperm pellet and on the composition of plasma from the cauda epididymidis of the rabbit

	4000 g	12,000 g	*Cold shock*+12,000 g
Spermatocrit (%)	40·99±10·14	19·22±3·7***	17·36±3·01***
Stained spermatozoa (%)	23·0±2·95	35·62±3·71	98·77±0·10***
Sodium (mequiv./litre)	15·0±1·09	14·91±0·96	14·74±1·12
Potassium (mequiv./litre)	22·17±1·54	22·26±0·49	22·55±1·15
Acid phosphatase (i.u.)	273·0±24·58	235·00±25·1	266·00±24·68
Alkaline phosphatase (i.u.)	13,730±1757·6	13,160±1707	12,160±1491·1
β-N-acetylglucosaminidase (i.u.)	17,750±3339·2	16,670±2408	13,200±1946·4
GOT (i.u.)	7·2±1·82	15·9 ± 3·12	1900±207***
LDH (i.u.)	1143±125	1676±322	33,528±1333***

Values given are the means for three animals with their standard errors. Centrifugation time = 5 min. i.u. = International Units (μmol substrate hydrolysed/min/litre at 37°C).

** Significantly different from 4000 *g* ($P<0·01$).
*** Significantly different from 4000 *g* ($P<0·001$).

There was a positive correlation between the incidence of stained spermatozoa and the levels of GOT ($r = 0·98$, $P<0·01$) and LDH ($r = 0·99$, $P<0·01$) in the supernatant plasma. The difference in GOT activity between 4000 *g* and 12,000 *g* was not significant ($P>0·05$) but the difference for LDH activity was significant ($P<0·01$). Cold shock and centrifugation at 12,000 *g* resulted in very high levels of GOT and LDH appearing in the plasma and these were significantly different from all other treatment groups ($P<0·001$). The levels of sodium, potassium, acid phosphatase, alkaline phosphatase, and *β-N*-acetylglucosaminidase in the plasma were not, however, significantly different between treatment groups ($P>0·05$), nor were they correlated to the incidence of stained spermatozoa in the spermatocrit.

Increasing the time of centrifugation at 12,000 *g* from 5 min to 10 or 20 min (Table 2) did not cause a significant decrease in the spermatocrit ($P>0·05$) although it caused a significant increase in the percentage of stained spermatozoa in the sperm pellet. The difference in the percentage of stained spermatozoa between 5 min and 10 min was just significant ($P<0·05$) but between

Table 2. The effect of time of centrifugation at 12,000 *g* on the composition of plasma from the cauda epididymidis of the rabbit

	5 *min*	10 *min*	20 *min*
Spermatocrit (%)	23·45 ± 0·7	20·0 ± 0·94	20·23 ± 0·72
Percentage of stained spermatozoa (%)	16·0 ± 0·57	25·0 ± 1·73*	37·0 ± 2·08**
LDH (i.u)	460·56 ± 42·2	501·2 ± 15·62	806·0 ± 21·9**

Values given are the means for three animals with their standard errors. i.u. = International Units (μmol substrate hydrolysed/min/litre at 37° C).

* Significantly different from 5 min ($P<0·05$).
** Significantly different from 5 min ($P<0·01$).

5 min and 20 min, it was highly significant ($P<0·001$). The levels of LDH in the plasma were significantly different between 5 min and 20 min ($P<0·01$) but were not significantly different between 5 min and 10 min ($P>0·05$).

The composition of epididymal plasma from the cauda epididymidis of normal intact animals is shown in Table 3. Here, the epididymal contents were centrifuged at 12,000 *g* for 5 min.

Table 3. The composition of plasma from the cauda epididymidis of the rabbit*

Characteristic or constituent	*Mean ± S.E.*	*No. of animals*
Spermatocrit (%)	20·43 ± 2·02	8
Sodium (mequiv./litre)	20·73 ± 2·33	5
Potassium (mequiv/litre)	24·80 ± 1·21	5
Magnesium (mequiv./litre)	14·02 ± 2·66	5
Chloride (mequiv./litre)	9·18 ± 0·99	5
Total protein (mg/100 ml)	4033 ± 227·7	5
Glycerylphosphorylcholine (mg/100 ml)	766·6 ± 41·59	5
Inorganic phosphorus (mg/100 ml)	12·42 ± 1·03	5
Fructose (mg/100 ml)	Undetectable	5
Osmotic pressure (ΔT_f,°C)	0·580 ± 0·035	5
pH	6·72 ± 0·16	5
Acid phosphatase (i.u.)	106·0 ± 41·13	5
Alkaline phosphatase (i.u.)	16,650·0 ± 1460·0	5
α-Mannosidase (i.u.)	912·0 ± 183·5	3
β-*N*-acetylglucosaminidase (i.u.)	21,890·0 ± 4741·0	6
Lactic dehydrogenase (i.u.)	648·47 ± 90·98	4

ΔT_f = depression of freezing point.
i.u. = International Units (μmol substrate hydrolysed/min/litre at 37° C).

* Epididymal contents centrifuged at 12,000 *g* for 5 min.

DISCUSSION

The epididymal contents collected by the cannulation procedure described in this work are free of contamination with blood or tissue fluid and are of sufficient quantity to allow a more extensive chemical analysis to be made than has hitherto been possible. It is enough to stimulate the cauda epididymidis only once or twice with the electrode in order to initiate vigorous contraction of the epididymal tubules. Continuous stimulation is unnecessary.

The present work confirms that the composition of epididymal plasma can be seriously affected by procedures that damage spermatozoa and cause leakage of their intracellular constituents. This problem is particularly relevant to epididymal contents where there is a high concentration of spermatozoa and where methods of separating spermatozoa other than by centrifugation are unsuitable because of the small volumes of fluid involved. The results do show, however, that whilst centrifugation at 12,000 *g* inevitably damages some spermatozoa, its effects can be reduced to a workable level by reducing the time of centrifugation to 5 min. Centrifuging epididymal contents at 4000 *g* for prolonged periods of time (20 min) was not a satisfactory alternative, because it did not result in maximum packing of spermatozoa in the sperm pellet and spermatozoa were therefore frequently found in the supernatant. The possibility that centrifugation caused a reduction in the volume of spermatozoa cannot be discounted entirely, but it is unlikely to account for a decrease in the spermatocrit of over 50% between 4000 *g* and 12,000 *g*.

Before any component of the epididymal plasma can be said to be truly extracellular, it is necessary to check for its release from spermatozoa either by prolonged centrifugation at high speed or after cold shock. On these grounds, it can be said that LDH and GOT originate in the spermatozoa, whilst sodium, potassium, acid phosphatase, alkaline phosphatase, and β-*N*-acetylglucosaminidase are truly extracellular. This finding is in agreement with the earlier observations of Graham & Pace (1967) on ejaculated bull semen, although these workers also found some evidence for the release of acid phosphatase as did Nath & Patt (1970) and Murdoch & White (1968) using ram spermatozoa that had been subjected to cold shock. In the present work, however, an increase in the activity of acid phosphatase was not detected, irrespective of the amount of damage to the spermatozoa, so it would appear that the amount of acid phosphatase released from the spermatozoa was insufficient to affect the total activity in the plasma.

The 1:1 ratio of sodium to potassium reported here in plasma taken from the cauda epididymidis of rabbits is in agreement with the results of previous workers using rams (Scott, Wales, Wallace & White, 1963; Jones, 1973), bulls (Crabo & Gustafsson, 1964; Wales, Wallace & White, 1966) and boars (Mann, 1964; Crabo, 1965). The rat and hamster appear to be exceptions since the ratios are 1:2·67 and 1:1·58, respectively (Levine & Marsh, 1971; Jones, 1973). The relatively high concentration of magnesium found in the epididymal plasma suggests that the epididymis may be an important source of this cation in the ejaculate, since rabbit semen also has high levels of magnesium (Quinn, White & Wirrick, 1965). The concentration of chloride ions by contrast, is similar to that reported by Crabo (1965) for bulls and boars and by Levine & Marsh (1971) for the rat.

The hypertonicity of epididymal plasma (0·58° C as against 0·57° C for blood plasma) is in agreement with that reported by Salisbury & Cragle (1956), Scott, Wales, Wallace & White (1963) and Levine & Marsh (1971), but the pH is much lower than that found by Bishop & Mathews (1952) for the luminal contents of the ductus deferens of the rabbit. Bishop & Mathews (1952) and White & Wales (1961) report values of 7·2 and 7·4 for the rabbit and ram, but

Branton (1946), Wales, Wallace & White (1966) and Levine & Marsh (1971) consider the pH of plasma from the cauda epididymidis of the bull and rat to be acidic. This may reflect species differences but a satisfactory answer to these discrepancies is still outstanding.

The presence of high levels of glycosidases and phosphatases in the epididymal plasma is in agreement with the earlier observations of Conchie & Mann (1957) and Murdoch & White (1968) on boars, stallions and rams. Conchie, Findlay & Levvy (1959) also reported the levels of α-mannosidase and β-*N*-acetylglucosaminidase in the epididymis of the rat to be higher than in any other mammalian tissue. Histochemical studies have shown that these enzymes are present in the epididymal cells lining the duct (Risley, 1963; Martan, 1969; Moniem, 1972) and it is possible that they are actively secreted into the lumen. However, morphological evidence for active secretion is still equivocal (Nicander, 1970; Moniem, 1972) and the origin of these enzymes is by no means clear. The only substance which is generally regarded as a specific secretory product of the epididymis is GPC (Dawson, Mann & White, 1957; Dawson & Rowlands, 1959; Scott, Wales, Wallace & White, 1963; Wallace, Wales & White, 1966). In the present study, the concentration of GPC in the epididymal plasma is approximately three times that reported in ejaculated rabbit semen (Mann, 1964), but it is considerably lower than in the epididymal plasma of the ram (Scott, Wales, Wallace & White, 1963; Jones, 1973) bull or boar (Mann, 1964; Crabo, 1965).

Thus, the *milieu* of spermatozoa in the cauda epididymidis is a highly specialized one and its significance in relation to sperm survival awaits further investigation.

ACKNOWLEDGMENTS

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ACCESSORY SEX STRUCTURES IN THE MALE NINE-BANDED ARMADILLO (Dasypus novemcinctus)

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The male armadillo is of especial interest in that, like the Cetacea, it not only belongs to the group of mammals known as the "testiconda", but the abdominal position of its testes is generally regarded as having developed secondarily from ancestral forms where the testes were situated in a scrotum. The present observations on two male nine-banded armadillos were made with the purpose of determining how far the male accessory organs of this species resembled those of mammals possessing a scrotum and whether any features might be present that could throw some light on sexual function in this species or on the phylogeny of its reproductive organs.

Gross dissection and histological examination of the organs revealed that the epididymis, whilst being coiled, was somewhat shorter than that of most mammals that have scrotally situated testes. Histologically, the head, body and tail of the epididymis could be identified and also an area equivalent to the initial segment was observed. The cauda epididymidis, however, was relatively small as a place of storage of spermatozoa. The vasa deferentia were typically mammalian in structure, but the ampullae did not appear to be markedly expanded.

A discrete compound tubulo-alveolar prostate gland was situated in two unconnected parts at either side of the pelvic urethra. Each part opened into the urethra by a series of ducts and had also produced a secretion containing PAS-positive material which was not removed by digestion with saliva at 37°C. The material stained well with Southgate's mucicarmine although it showed only indistinct metachromasia when stained with toluidine blue. PAS-positive granules could also be seen in the glandular epithelium. The bulbo-urethral glands, which had large bundles of skeletal muscle between the lobules, showed similar staining properties to the prostate.

The seminal vesicle appeared as two large conjoined sacs but its particular interest was in relation to what seemed at first glance to be its ducts. These were shown to be merely a continuation of the vesicle itself, extending under cover of urethral muscle and opening in the caudal third of the urogenital sinus, together with the vasa deferentia. The vasa deferentia and the "ducts" of the seminal vesicle never joined so that true ejaculatory ducts were not evident.

The pelvic urethra was long and lined with transitional epithelium. Ventrally, it appeared to possess a large diverticulum which extended cranially almost to the level of the prostate. This was not visible from gross dissections. Microscopically, it was seen to be packed with spermatozoa. Particular attention was called to this rather unusual feature.

Observations on the structure of the penis concurred in

large measure with the descriptions given by Newfang (J. Morph. 1947, 81) and Buchanan (Anat. Rec. 1960, 136) No prepuce was evident and teleologically, therefore, it might not seem surprising that a retractor penis muscle could not be discerned.

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The reproductive system of male rock hyrax (*Procavia* and *Heterohyrax*)

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(With 6 plates in the text)

The male genitalia of the rock hyrax (*Procavia* and *Heterohyrax*) are described. Features of the gross anatomy and histology of the testes and male accessory organs are presented, and comparison is made between the sexually active and sexually quiescent animal.

Microscopical observations have revealed the main area of storage of spermatozoa in this mammal and partly on these grounds, a new nomenclature for different parts of the reproductive system is proposed. Seasonal variations in male sexual activity in these mammals are also discussed, in addition to anatomical considerations.

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Introduction

Although the testes in most eutherian mammals are situated in a scrotum, it has long been recognized that in some species they are permanently and normally intra-abdominal. This is a usual feature of prototheria, and Owen (1856), referring to the monotremes, wrote of them as "true testiconda". Later, Weber (1927) explained the term as referring to those mammals whose testes are always intra-abdominal, and where, in addition, the inguinal canals and inguinal ligaments are absent. Some eutherian mammals fall into this category, and the Hyracoidea are among them, together with certain families of Insectivora, Edentata, Proboscidea, and Sirenia.

The question of testicular function in the "testiconda" is interesting in view of the extreme sensitivity of scrotal testes to the effects of abdominal temperature, and it is reasonable to enquire how sperm production is achieved in these mammals, since it presumably occurs at abdominal temperature. A search for any structural peculiarities that might help to

explain this seemed to be the obvious first step in looking at the problem, and anatomical findings provide the main substance of this paper.

Several descriptions of the genital organs of "testiconda" already occur in the literature (Owen, 1856; Oudemans, 1892; Weber, 1898), with a fairly detailed account on the hyrax by George (1874). Hitherto, reference appears to have been confined to gross anatomical features, however, and closer scrutiny was called for if any special functional characteristics were to be revealed. Of the true "testiconda", the rock hyrax is quite a suitable animal for detailed investigation, both in terms of its size and availability. It is for this reason that the authors have initially paid more attention to it than to other African "testiconda".

Materials and methods

Material was obtained from 12 male hyrax, of which 3 were *Heterohyrax* and 9 were *Procavia*. Both genera are small herbivores sheltering in existing holes in rocky outcrops throughout much of Africa (Sale, 1960, 1966) and the Middle East (*Procavia* only). Unlike the Tree hyrax (*Dendrohyrax*), to which *Heterohyrax* is closely related, both the rock forms are social and live in colonies, which vary in size from 6 to 100 or more animals. The majority of colonies contain only one genus, but cases are known where the two genera share the same group of rocks (Turner & Watson, 1965). There is considerable overlap in habitat and food selection (Sale, 1965*a*) and the diurnal behaviour cycles of the two forms are also very similar, a marked feature being the large amount of time they spend basking in the sun (Sale, in press).

Procavia is the larger of the two genera, and the mature males obtained for this work weighed from 2·5 to 3·1 kg, although one immature animal weighed only 1·2 kg. The weight of the *Heterohyrax* males was 1·3 to 1·7 kg. Some of the *Procavia* were found on escarpments in the Ngong area, not far from Nairobi, whilst the remainder and all the *Heterohyrax* were obtained in the Nanyuki district, 120 miles further north on the equator.

Immediately after death, each animal was weighed on a spring balance and the reproductive organs were then removed and weighed. In 2 animals, the tract was simply dissected clear of surrounding tissue and photographed, whilst in 8, different regions of the tract were fixed in formal saline (10% formalin in normal saline). The tract of one animal was fixed in Bouin's fluid, but formal saline was preferred since it allowed more versatility in subsequent staining methods. It was found to be necessary to take small pieces of tissue on the spot, for it had previously been found that if the whole tract was placed in fixative and detailed fixation of small pieces was left for some hours or days, fixation of some of the deeper tissues was quite inadequate. This is mentioned, bearing in mind the conditions of work in the African bush, where it is tempting to delay detailed work until returning to the laboratory. In this context also, it is recommended that fixatives always be kept as cool as possible.

An attempt was made to obtain spermatozoa from proximal and distal levels of the tract in each animal, and when this was possible, samples were examined subjectively for motility, and a sperm smear stained with aqueous nigrosin eosin was also made.

Histological sections were stained routinely with Harris' haematoxylin and eosin, and since the amount of plain muscle around the excurrent ducts of the testes was of interest, Van Giesen's stain was also used. Other than reference to the use of Sudan black (Threadgold, 1957), histochemical investigations are not included in this paper.

Anatomical observations of the reproductive tracts soon confirmed that there were marked differences between mature males in the degree of sexual activity, suggesting the existence of breeding cycles in these animals (Sale, 1965*b*). Testis weights were therefore taken, together with measurements of the length of the testes, in order to have some estimate of the level of sexual activity of individuals. For convenience of description, animals will be referred to as being "sexually active" or "sexually quiescent", although it is evident that intermediate stages of sexual

activity occur in adult males. Sexually quiescent males, however, are considered here as those in which no significant spermatogenic activity is taking place.

Of the 12 animals from which this material was obtained, the smallest one was definitely immature, as shown by the fact that the third upper molar had not yet begun to erupt. Only 3 (2 *Heterohyrax* and 1 *Procavia*) could be regarded as sexually active, and these were all killed in the month of September. The remainder were killed in the latter half of July and during August.

Gross anatomical features of the genitalia

Dissection and observation of the topography of abdominal viscera in the hyrax shows that the left testis is situated caudal to the right (Plate I). Although there is not an excessive difference in position, it is distinct and not related to the position of the kidneys which are symmetrically aligned, in spite of asymmetry in the origin of the renal arteries. There is, however, an association of each testis with the ipsilateral kidney through a firm peritoneal attachment. Moreover, the left testicular artery originates from the inferior renal artery (there are three arteries to each kidney) and the right testicular may also come off the renal when it does not arise from the aorta close to the point of origin of the right inferior renal. The testicular arteries are virtually straight and there is no evidence of a pampiniform plexus of veins around the arteries.

The weight of the testes in sexually quiescent animals varied from 1·0 to 1·65 g and their length varied from 2·2 to 3·1 cm. Differences in testis length were seen to be directly related to body weight in these animals. In sexually active animals, however, the weight and size of the testes did not necessarily appear to be related to the size of the animal. Nevertheless, in two animals, one *Heterohyrax* and *Procavia*, the testis weights were 6·5 and 8·6 g respectively, with lengths of 4·5 and 4·6 cm. In one sexually active *Heterohyrax*, which was admittedly only 1·3 kg in weight, the testes weighed only 1·65 g and were 2·9 cm in length. These data represent mean weights and lengths, but there was virtually no discrepancy in weight or length between contralateral testes. It is therefore clear that increased weight and size of the testes in the sexually active state is very considerable, and it may be unmistakable even by casual observation, as in one case, where the testes practically filled the entire dorsal part of the abdomen.

Detailed examination of the ductuli efferentes in this species has not yet been undertaken, but they can be seen emerging relatively discreetly from the dorsal pole of the testis into the proximal end of the Wolffian duct. Remaining accessory structures are illustrated in Plate I, with a new nomenclature based on findings given in this paper.

The Wolffian duct is extensively coiled as it runs along the length of the testis, after which it straightens out as it passes over the ureter to reach the pelvic cavity. At the entrance to the pelvis, the duct becomes confluent with its contralateral partner and is seen once more to be excessively coiled. The coils here are not easily discernible in the sexually quiescent animal, and the two adjacent ducts give the appearance of a small sac-like structure. They are visible, however, in sexually active males (Plate II), when the bulbous form of the combined ducts appears to descend well into the pelvic cavity, presumably due to the weight of spermatozoa packing them at this level. This coiled region is covered with a network or lattice of closely adherent peritoneal connective tissue (Plate V(c)) and the region was referred to by George (1874) as the "seminal vesicle". It empties its contents into the pelvic urethra through short straight or "S"-shaped ducts.

Also emptying into the pelvic urethra are two large tubular glands which, in gross

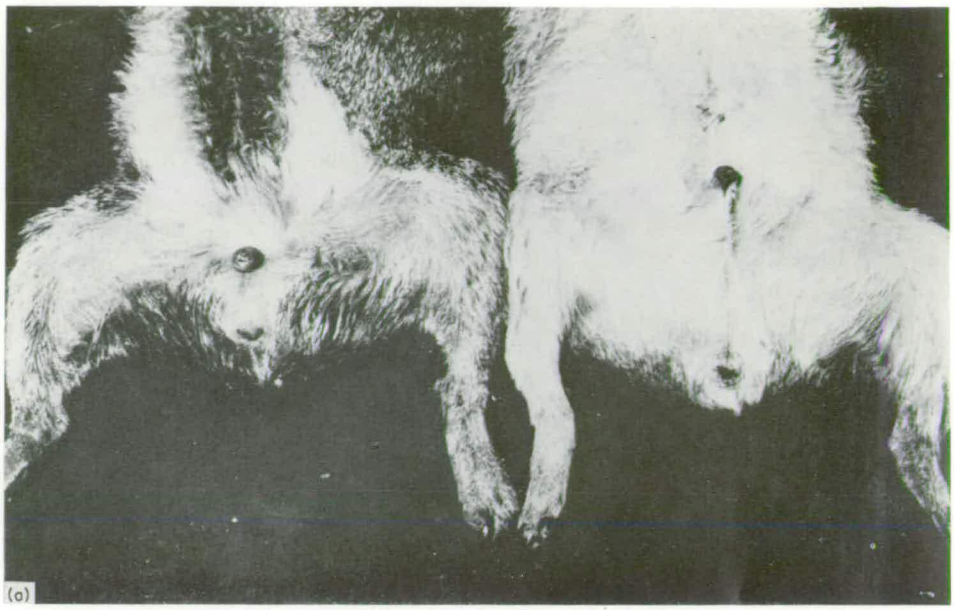
appearance, typify the seminal vesicles in some other eutherian mammals, but which are referred to by George (1874) as the "prostates". In the sexually active animal when the coiled sac-like part of the Wolffian duct descends into the pelvic cavity, it moves caudal to the main body of this tubular gland. More caudal still, there are two small conical structures symmetrically situated on either side of the urethra. These, George (1874) designated "Cowper's glands". Histological examination of these different organs was undertaken to test the validity of George's terminology, which was adopted by Grassé (1955), since precise identification from their gross structure alone might well be unreliable.

The penis in both *Procavia* and *Heterohyrax* is of the vascular type, where erection is produced primarily by vascular engorgement of the cavernous tissue. The corpus cavernosum penis has no septum, although it is invaded by substantial connective tissue trabeculae (Plate II(b)). Close to the root of the penis the corpus cavernosum penis appears to be characterized by the presence of considerable muscular tissue, but this diminishes in the body of the penis. Cavernous tissue extends dorsolaterally beyond the external urethral orifice in the form of two small bulbs. Here also, the median raphé of the penis is particularly distinct. The penis of *Heterohyrax* is distinguishable from that of *Procavia* by a short, straight and filiform appendage, which endows the end of the penis with a fleur-de-lys appearance. This is quite a useful feature for rapid identification. In addition, the position of the preputial orifice differs in the two species, as illustrated in Plate II(a). In *Procavia*, the average distance between the anus and the end of the prepuce is 24 mm, whilst in *Heterohyrax* it is about 65 mm. This difference has already been considered as a feature of some taxonomic significance in South African hyrax (Coetzee, 1966).

The muscles of the penis in hyrax are unusual in gross form, and description is made difficult due to the penis pointing downwards or backwards when not erect, or partially erect. Apparently, when the penis is merely extruded (semi-erect), it points backwards and projects posteriorly below the anus. In this respect, it is remarkably similar to the penis of the rhinoceros. Thus the borders conventionally regarded as being ventral (such as the median raphé of the penis) are in fact caudal or even dorsal in position. The situation is reversed in erection when the penis swings forward adjacent to the ventral abdominal wall. The large muscle mass mentioned earlier and shown in Plate I, is therefore best regarded as the bulbo cavernosus muscle, since it will come to lie caudal or ventral to the root of the penis in erection. It is a compact muscle and does not run along the ventral surface of the penis as is typical of most eutherian mammals. It is not perhaps surprising though, that it seems to increase in size in the sexually active animal. A smaller muscle of the penis is shown in Plate I(a): this bilateral muscle is situated at the crus, on what will be the dorsal surface of the penis in erection. The tendons of the muscle fuse to form a strong single tendon which extends throughout most of the length of the body of the penis. This tendon is also illustrated in Plates I(a) and II(b) and is plainly concerned with pulling the penis forward in erection. The muscle and its tendon are therefore best regarded as the ischio cavernosus.

Histological features of the testes and accessory sexual organs

Histological sections of the testes are shown in Plate III. Different stages of spermatogenesis could be recognized in all three sexually active animals, and many tubules showed spermatozoa attached to Sertoli cells. Fixation of testes in formal saline seemed to give



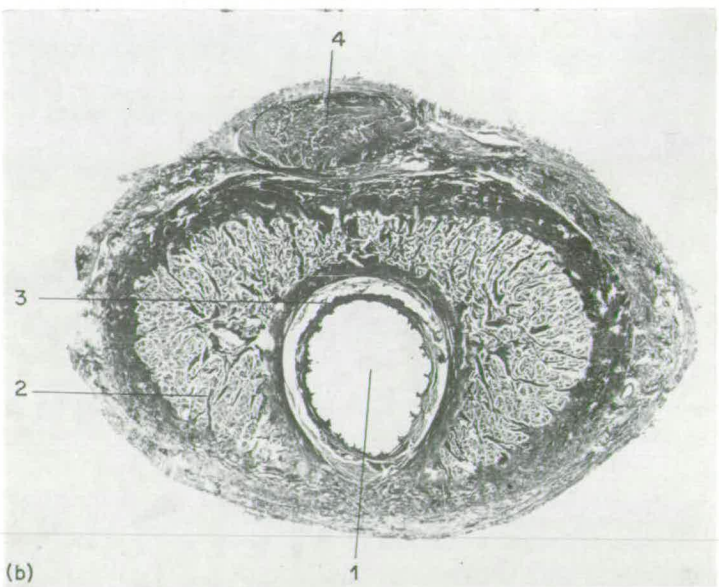


PLATE II. (a) Inguinal region of hyraces showing generic difference in position of preputial opening. Left, *Procavia;* right, *Heterohyrax.*

(b) Section of penis of *Procavia.*

1, Urethra; 2, corpus cavernosum penis; 3, corpus cavernosum urethrae (corpus spongiosum); 4, tendon of ischio–cavernosus muscle.

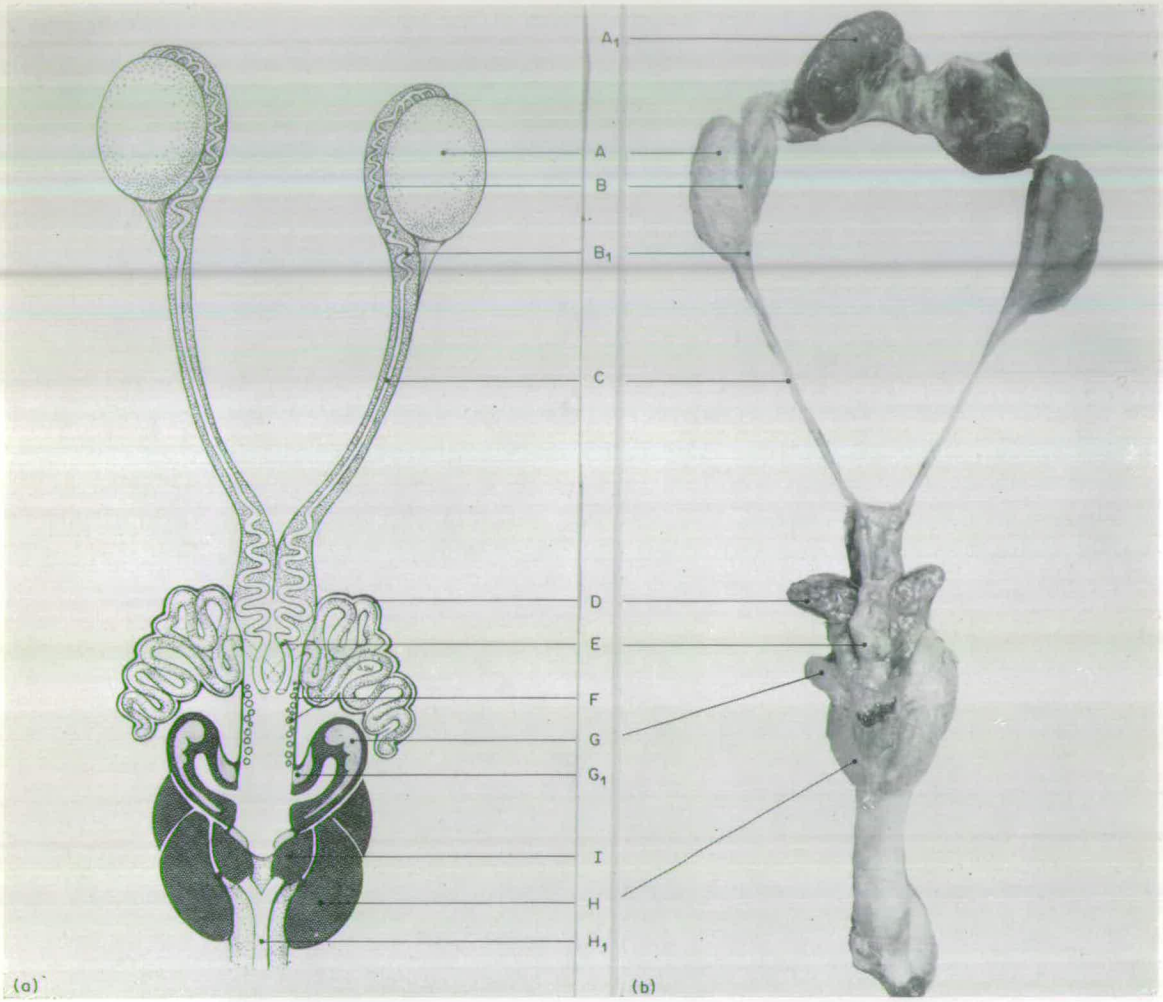


PLATE I. Photograph and corresponding diagram of male reproductive tract of rock hyrax, illustrating the testis and accessory sexual structures with suggested nomenclature. (See Discussion in text.)

A, Testis; A_1, kidney; B and B_1, caput epididymidis; C, corpus epididymidis; D, seminal vesicle; E, cauda epididymidis; F, prostatic tissue; G, Cowper's (bulbo-urethral) gland; G_1, Cowper's ducts; H, bulbocavernosus muscle; H_1, tendon of ischiocavernosus muscle; I, ischiocavernosus muscle on crus of penis.

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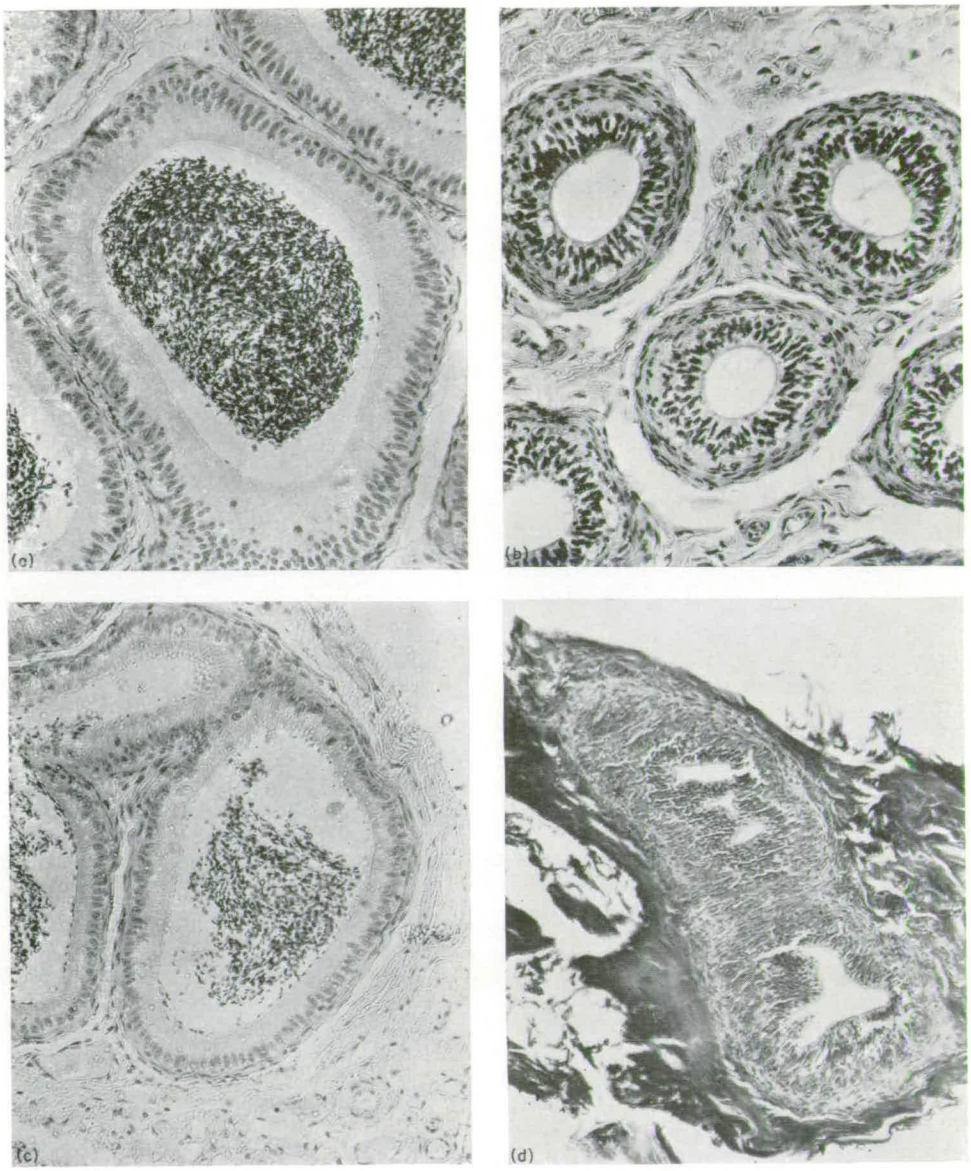

PLATE IV. (a) Histological appearance of the epididymis, adjacent to the distal pole of the testis in a sexually active male, showing resemblance to the head of the epididymis in other species.

(b) Histological appearance of the epididymis adjacent to the distal pole of the testis in sexual quiescence. This section was taken at the same magnification as (a) and illustrates stratification of the epithelium, thickening of the muscular wall and complete absence of spermatozoa.

(c) Histological appearance of the straightened part of the Wolffian duct in a sexually active male showing the presence of spermatozoa and fairly tall epithelium.

(d) The straightened part of the Wolffian duct in a sexually quiescent male at a lower magnification, especially to illustrate thick muscular wall (stained with Van Giesen). (a), (b), (c) $\times 160$; (d) $\times 64$.

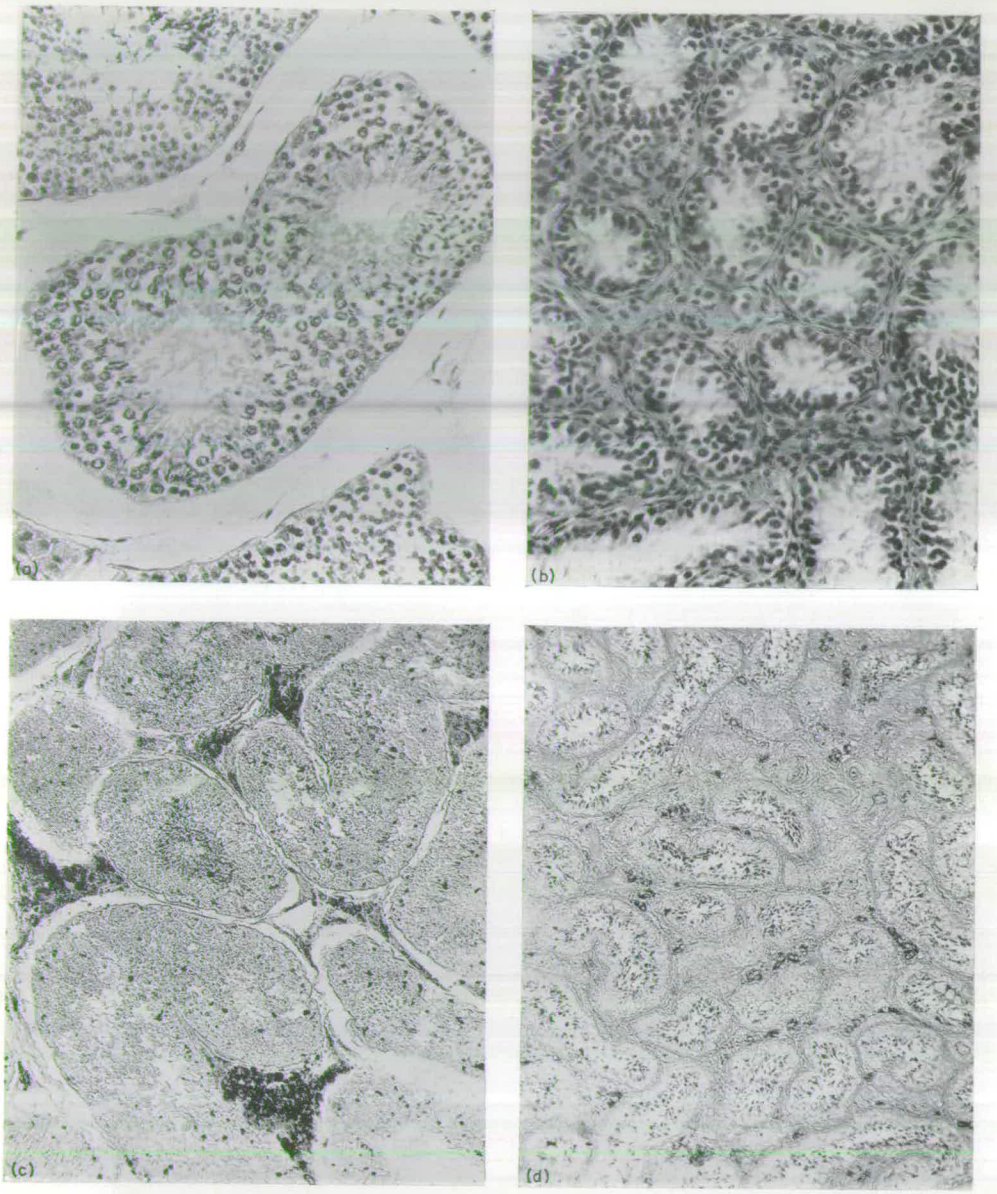

PLATE III. (a) Seminiferous tubules of a sexually active hyrax showing spermatogenic activity.
(b) Testis of a sexually quiescent male showing complete interruption of spermatogenesis, pyknosis of nuclei, and occasional "phytoid" spermatogonia.
(c) Section of active testis stained with Sudan black showing extensive distribution of interstitial tissue.
(d) Section of testis in sexually quiescent male showing marked reduction in amount of interstitial tissue.
All $\times 120$.

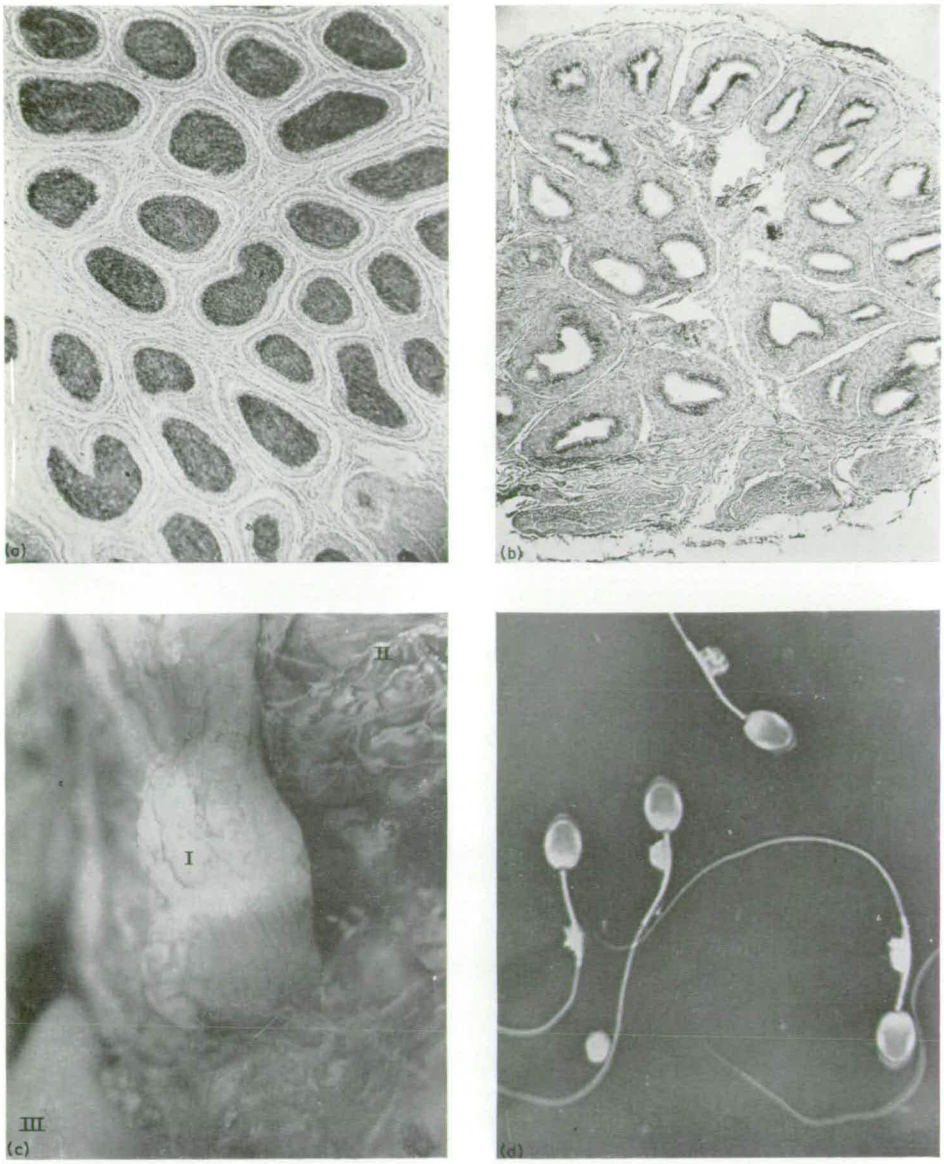

PLATE V. (a) The distal coiled area (E) of the Wolffian duct of a sexually active hyrax illustrating that this is the main storage centre for spermatozoa in the rock hyrax. The duct is seen to be packed with spermatozoa.

(b) The distal coiled part of the Wolffian duct in sexual quiescence showing complete absence of spermatozoa a structure similar to the tail of the epididymis in other mammalian species.

(c) Gross appearance of the sperm store (E) (distal coiled region of the Wolffian duct) showing the net-like peritoneal covering and the general resemblance of the area to the tail of the epididymis. I, Wolffian duct (cauda epididymidis); II, seminal vesicle; III, Cowper's (bulbo-urethral) glands.

(d) Spermatozoa taken from sperm store in the Wolffian duct. Stained with aqueous nigrosin eosin. (a), (b) ×64; (d) ×960.

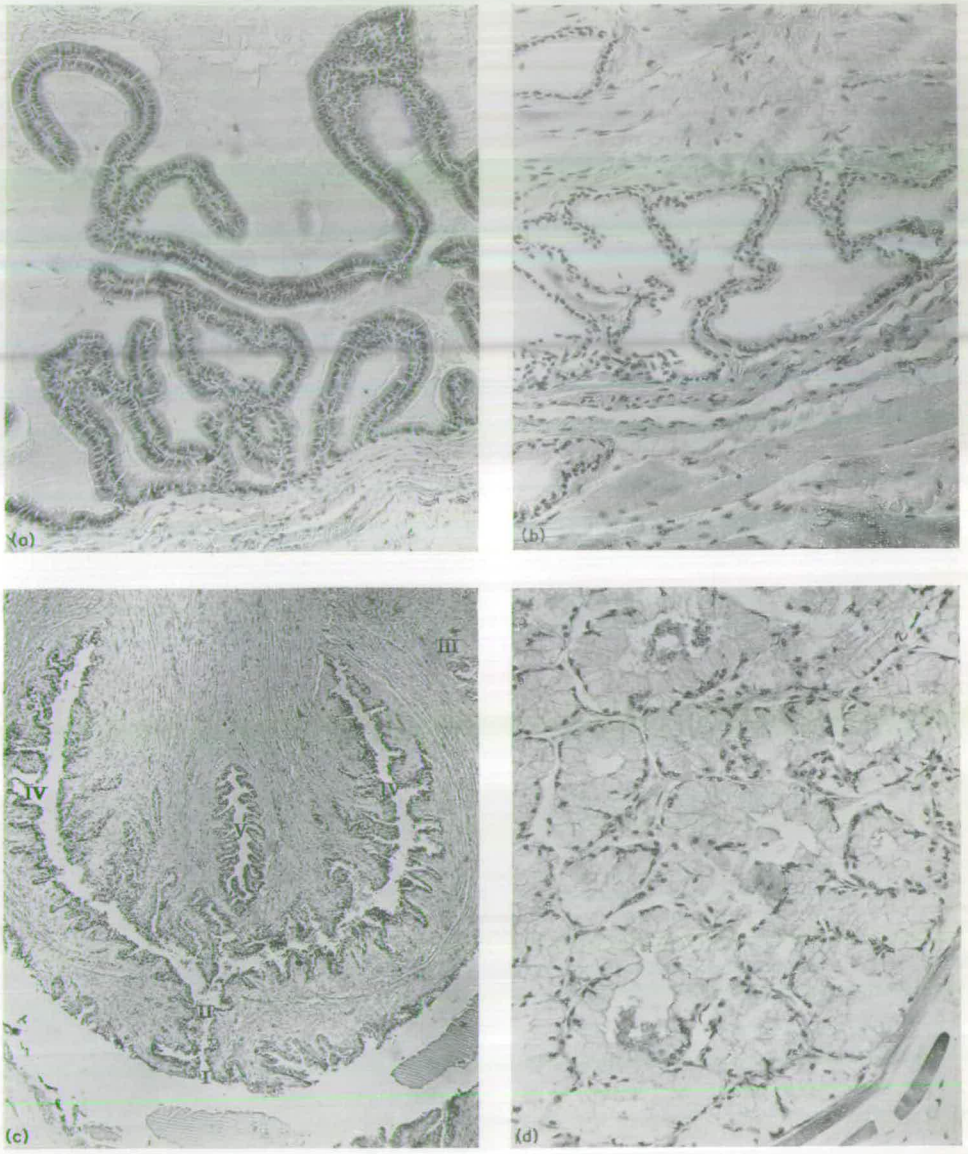

PLATE VI. (a) Histological section of organ suggested as seminal vesicle.

(b) Prostatic tissue in wall of urethra.

(c) A section of prostatic urethra. I, Urethral crest; II, prostatic ducts entering urethra; III, ductus deferens on one side; IV, prostatic ducts; V, utriculus prostaticus (uterus masculinus).

(d) Cowper's (bulbo-urethral) tissue with surrounding skeletal muscle. (a) ×120; (c) ×64; (b), (d) ×160.

rise to considerable shrinkage, but in spite of this, typical interstitial tissue was seen between the tubules.

Curiously, there appeared to be less shrinkage in sections taken from the testes of sexually inactive animals. Here, spermatogenesis was obviously arrested and there was a complete absence of spermatozoa in the seminiferous tubules which were much reduced in size. The picture was characterized by condensation and pyknosis of nuclei in the spermatogonia and spermatocytes, numerous bizarre mitotic figures in the remaining spermatocytes, and several "phytoid" spermatogonia (Harrison, 1956) where the cytoplasm of the cells seemed to be replaced by a large vacuole. Haematoxylin and eosin preparations gave the impression that the interstitial tissue was more abundant than in the testes of sexually quiescent animals. Since this seemed unlikely, however, further sections were stained with Sudan black. These showed that interstitial tissue was, in fact, much more extensive in the sexually active testes (see Plate III(c), (d)), and it was clear that the appearance of haematoxylin and eosin sections was, in this context, illusory.

Sections of Wolffian duct taken from a level at the equator of the testes in sexually active males (Plate I,B) revealed a tubular structure akin to that of the head of the epididymis in most eutherian mammals (see Plate IV(a)), although the stereocilia were rather less defined than is usual for the area. The epithelium was quite high and markedly pseudostratified. A thin layer of plain muscle surrounded the duct at this point, as confirmed in sections stained with Van Giesen. The duct was packed with spermatozoa. It is important to note that at a level opposite the distal pole of the testis (Plate I,B_1) the histological structure of the duct was virtually unchanged.

In sexually quiescent animals, these levels of the Wolffian duct showed excessive stratification of the epithelium and an apparent reduction in supranuclear cytoplasm (Plate IV(b)). The lumen of the duct was greatly reduced and devoid of spermatozoa, and the surrounding layer of plain muscle appeared to be thicker than in sexually active animals.

Sections of the straightened part of the Wolffian duct (Plate IV(c)) showed it to have a slightly different structure from more proximal levels. The epithelium was lower and there was less pseudostratification. Stereocilia were rather more apparent and secretion blebs were clearly visible. Surrounding muscle was slightly thicker than in more proximal levels and in the sexually active animal the lumen was again filled with spermatozoa. In the sexually quiescent state, the lumen was very much reduced, spermatozoa were entirely absent and the muscular wall was so thick as to occupy most of the tubule diameter (Plate IV(d)).

The histological structure of the coiled distal end of the epididymis (Plate V(a)), that is, in the region referred to by George (1874) as the "seminal vesicle", differed from that at more proximal levels in that the epithelium was much lower, was not pseudo-stratified and had virtually no stereocilia. Coils appeared to be gathered together in lobules by intervening connective tissue trabeculae, and this was particularly apparent in the sexually quiescent animals (Plate V(b)). Here also, the duct was surrounded by a considerable thickness of plain muscle, which, as at more proximal levels, seemed to thin out in the sexually active state, when the lumen was tightly packed with spermatozoa.

The structure of this area in the sexually active and quiescent conditions bears a striking resemblance to the tail of the epididymis in active and aspermic mammals with scrotal testes. The same basic structure persists, as the duct straightens out again to enter the pelvic urethra as short ducti deferentes.

A section of the bilobed gland (Plate I,D) hitherto known as the prostate (George, 1874; Grassé, 1955) is shown in Plate VI(b). This was taken from a sexually active male and it is evident that its structure is typical of a seminal vesicle. It is a simple tubular gland with columnar epithelium and in the sexually active state, it is filled with large amounts of relatively homogeneous secretion. The ducts from the gland enter the urethra from its dorsal aspect and shortly before entry, the septum between the two ducts ends, so that the secretions are apparently poured into the urethra through a single opening. This occurs, it seems, just before the excurrent ducts from the testes open into the urethra, so a true ejaculatory duct, of the type occurring in primates, is not formed.

On simple histological grounds, it seems that in the hyrax, prostatic tissue (Plate VI(b)) does not occur as a discreet gland but is disseminated along the walls of the pelvic urethra (Plate I,F). Sections through this prostatic part of the urethra show typical prostatic ducts situated dorsal to the urethra alongside a utriculus prostaticus (uterus masculinus) (Plate VI(c)).

The conical structures which George (1874) called Cowper's glands, make an interesting picture (Plate I,G). In sexually active hyrax, they appear distinctly hollow, although the central cavity seems to collapse somewhat in sexual quiescence, giving them an altogether more solid appearance. In sexually active animals, the cavity appears to be lined with striated muscle, which also covers the whole structure, but the main substance of the structure is represented by glandular tissue which is typical of bulbo-urethral glands (Plate VI(d)). There is a small area in the anterior wall of the gland (Plate VI(b)), which is separated from the main mass of the Cowper's tissue by a band of striated muscle (see Plate I). This area has scattered glandular tissue but is mostly occupied by ducts that presumably represent the main outlet of the Cowper's glands into the urethra. Also, there are clumps of glandular tissue lining the urethra, as far down as the crus of the penis, and histologically, this closely resembles Cowper's tissue.

No spermatozoa, other than an odd disintegrated form, were obtained from sexually quiescent animals, but this was in marked contrast to material from sexually active ones, where sperms could be easily obtained from all levels of the Wolffian duct. From proximal levels of the duct (B and B_1), adjacent to the testes, fewer sperms could be obtained than from more caudal levels, and, in addition, their motility *in vitro* was negligible. Spermatozoa taken from the pelvic part of the duct (E), however, were vigorously motile.

With the light microscope, the spermatozoa appeared to be typically mammalian in structure but they had fairly small rounded heads and quite long tails (Plate IV(d)). There appeared little evidence to support the statement of Tuzet, Vogeli & Manier (1957) that hyrax spermatozoa are not of the classical mammalian structure and show primitive characters similar to those of lower vertebrates, The fact that the single specimen mentioned by these workers was of the genus *Dendrohyrax* is unlikely to explain the peculiarities observed, and it seems more probable that it was a sexually quiescent animal with disintegrated forms of spermatozoa, or that it was abnormal.

In the present work, protoplasmic droplets were seen to have been discarded or to have migrated to the end of the midpiece in most of the spermatozoa taken from level E (Plate I). This was in contrast to those taken from more proximal levels. This suggests that a process of maturation was occurring in the duct, but the phenomenon of sperm maturation in this species needs further study.

The incidence of sexual activity

From an examination of the testes and reproductive tracts of a large number of specimens (including the sample used for the present studies), it is clear that the male hyrax falls into one of three reproductive categories. There is the immature or juvenile animal which, as well as being small in size, has incomplete tooth eruption and shows no evidence of spermatogenesis. Of the animals of adult size and dentition, the majority are in a sexually quiescent state, with small flaccid testes and never more than a few disintegrated forms of spermatozoa. The third category consists of a small number of sexually active adults, in which the testes are large and swollen and all levels of the Wolffian duct contain numerous spermatozoa. A further characteristic of this last group is that the dorsal gland in the centre of the back shows much greater secretory activity than that of sexually quiescent males (Sale, 1965*b*) Also, there are frequently fresh external wounds on the back and shoulders of sexually active animals, indicating recent fights with other males.

The sexually inactive juvenile animal is to be expected, but the occurrence of two categories of sexual activity in the adult male raises some interesting questions. Clearly, all mature male hyrax are not continuously capable of reproduction, but it is possible that certain adult males are sexually active the whole year round, whilst others remain indefinitely in a state of sexual quiescence. Evidence from behavioural observations and breeding data, however, suggest that this is not true. Outside the tropics, hyrax have a definite breeding season corresponding with the local spring. (For example in Israel: see Mendelssohn, 1965, and South Africa: see Brand, 1963.) Mendelssohn (1965) states that during the mating season, Syrian hyrax (*Procavia* sp.) males make their territorial call and become very intolerant of other adult males. This suggests that these male hyrax are only sexually active during the mating season, internal changes being evidenced by the onset of a specific vocalization and fighting with other males. It is not clear, though, whether all adult males become sexually active during this period, but Mendelssohn makes the significant point that "Some adult males may, even during the mating season, tolerate the presence of sexually mature, sixteen-month-old males, but will never tolerate the presence of twenty-eight-month-old or older males". Does this mean that the young (16 month) males are, in fact, not sexually active but that all the older males are both sexually mature and active?

In the tropics, many species of hyrax do not have a well defined breeding season (O'Donoghue, 1963; Sale, in prep.). Observations on behaviour of rock species, however, indicate that mature males have only limited periods of sexual activity. In the Mount Kenya hyrax (*Procavia* sp.), for example, most males appear to have two periods of about three months' duration each year, when they make the territorial call and give other indications of being sexually active. Known instances of copulation have taken place towards the end of these periods, during which the degree of activity builds up to a peak, and then stops fairly abruptly. Unlike the non-tropical species, the periods of activity of individual males are not co-ordinated, so that at any time of the year in a large population, a few sexually active males are always likely to be found. This is confirmed by the examination of gonads over a number of years in several different populations (Sale, unpublished). Sometimes, it seems that there are seasons of exceptionally widespread sexual activity in a population and conversely, there may be periods of very little sexual activity. These periods correspond with seasons of high and low birth frequency respectively. Thus O'Donoghue (1963)

found "that although breeding is not restricted to any particular season in *Dendrohyrax*, the frequency of births is highest in the middle of the year".

Whilst the length of individual periods of activity, and the relative scarcity of active males in tropical populations at any one time, would both allow of all the adult males regularly undergoing periods of sexual activity, there is some evidence that certain males remain quiescent for indefinitely long periods or even permanently. The rock hyraces have a social system similar to that of some ungulates, for example, the Impala antelope, wherein the basic breeding unit is a polygynous herd consisting normally of one fully adult male, a number (varying from three to ten) of adult females and immature of both sexes. There is no evidence for a disparity between the sexes in the newborn young and although there is often differential predation on the males, there is usually a "surplus" which form themselves into bachelor herds. A group that contains gravid females nearing parturition forms a "nursery" herd from which all adult males are excluded (Sale, 1965*c*). When an adult male separates from his harem, often he does not join a bachelor herd but becomes a lone male wandering some distance from his females, but keeping within a limited range of them. It seems that such "attendant" males often re-enter the same group of females at the end of the nursery period. If this is a regular occurrence, members of a bachelor group only get an opportunity to have an harem of their own when the old male in a polygynous group dies or becomes too weak to fight off the bachelor seeking to take over.

That all members of bachelor herds undergo regular periods of full-scale sexual activity would seem unlikely, since this might lead to much destructive fighting, especially if two or more males were active concurrently. Moreover, the apparently continuous association of a sexually mature male with a group of females, suggests that while this male undergoes regular cycles of sexual activity, at least some members of a bachelor herd might remain quiescent for long periods. The mechanism responsible for such prolonged quiescence is not definitely known but a clue to it may be found in the fact that in captivity, it has been observed that the presence of one sexually mature (and periodically active) male tends to inhibit the development of sexual activity in other males in the same group. The removal of such a sexually mature and active animal is often followed within a short time by the onset of sexual activity in another mature male in the group. What cannot be established from observations of this kind is the relationship between hormonal levels and the state of the testes and the animal's behaviour. It is, of course, possible that only behavioural inhibition is taking place while regular cycles of spermatogenic activity are going on all the time.

Discussion

The peritoneal attachment of the testes to the kidney and the absence of inguinal canals in all the animals examined, supports Weber's contention that *Procavia* are "true testiconda". There is little doubt, moreover, that this applies to all the Hyracoidea. An especially interesting feature of male rock hyrax, however, is the striking increase in testicular size associated with behavioural evidence of periods of sexual activity. The changes in size and weight of the testes are so dramatic that they can be compared with those of avian testes. Like avian testes also, and unlike those of the elephant, the testes of hyrax are closely bound to the dorsal body wall.

Increased interstitial tissue in the testes of sexually active animals combined with spermatogenesis in the seminiferous tubules, suggests that under certain conditions the testes of some hyrax become especially responsible to gonadotrophins or that the level of circulating gonadotrophins is heightened. It should be possible in the future, to determine the true nature of this situation.

Earlier descriptions of the male reproductive tract of hyrax have utilized classical terminology and referred to that part of the Wolffian duct that lies on the surface of the testis, as the epididymis, and named the rest of the duct, the vas deferens. Pallas (1766), however, in his original description of the hyrax comments that the distal coiled region of the ductus deferens "*secundarium quasi epidydimidum constituunt*". In other words, he states that it virtually constitutes a secondary epididymis. The histological structure of the so-called epididymis is strongly reminiscent of the head of the epididymis only, and even at a level opposite the distal pole of the testis (Plates II and I, B_1), the duct bears little resemblance to a typical cauda epididymidis. Throughout the course of the duct on the testis, the epithelium is high and possesses stereocilia, thus being unrepresentative of distal levels of an epididymis in a typical eutherian mammal, where the epithelium is usually cuboidal in type and stereocilia are particularly sparse.

It is not surprising, however, that the remainder of the Wolffian duct has hitherto been called the vas deferens (Grassé, 1955), not only because of its topographical position but also because if it is examined in sexually quiescent animals, its histological structure is akin to that of the ductus deferens in other eutheria. The muscular wall, for instance, is thick and the lining epithelium is slightly lower than at more proximal levels. Nevertheless, in sexual activity the lumen is larger and the surrounding muscle layer becomes quite thin. This suggests that the muscular wall is not substantial and is capable of yielding to pressure from a lumen packed with spermatozoa. The only other explanation, which can surely be discounted, is that muscular tissue disappears in the breeding season. Basically, therefore, since the structure of the tract during the breeding season seems the most legitimate for description, the straight part of the Wolffian duct would seem better considered as the homologue of the body of the epididymis rather than the ductus deferens. There is evidence from accounts of other species that this may be feasible, for example, in *Elephantulus* this straight part of the duct has been designated the corpus epididymis (Stock, 1954).

If this be accepted, then the coiled part of the distal end of the duct, which George (1874) named the seminal vesicle, must be regarded as the true cauda epididymidis. This is acceptable, not only on the basis of its histological structure, but also on the fact that in sexually active animals the coiled duct is crammed with spermatozoa that are vigorously motile *in vitro*. The motility of spermatozoa from this level contrasts markedly with that of spermatozoa from more proximal regions, which indicates that a process of sperm maturation is occurring in the straight part of the duct. Morphology of the spermatozoa tends to support this, though an assertion at this stage would be premature.

If we continue to adhere to the older nomenclature, therefore, and try to translate it into functional terms, it must be claimed that in the rock hyrax, maturation of spermatozoa takes place in the ductus deferens rather than in the epididymis, and the seminal vesicles form a direct extension of the ductus deferens and serve as the main store of mature spermatozoa. Such conditions are so atypical of eutherian mammals as to be unlikely, and it is suggested that a different nomenclature be adopted, (as presented in Plate I). This would avoid the arrangement of male genital organs in the hyrax being regarded as fundamentally

different from those of other eutherian species. Whether this nomenclature can be applied to the testiconda generally, is difficult to say, but histochemical studies in hyrax are at present in progress to provide more functional information in at least one order, and other species might be usefully examined from this point of view.

The suggested nomenclature does mean that the ductus deferens of the rock hyrax is short and does not have a particularly thick muscular wall as in mammals with scrotal testes. This might be expected, however, if it can be accepted that in the hyrax, the tail of the epididymis (Plate I,E) is relatively close to the pelvic urethra. Thus, in seminal emission (Cross & Glover, 1958), the spermatozoa of hyrax need not travel so far as when the testes are in a scrotum and the sperm stores are thus further from the pelvic urethra.

If the distal coiled region of the Wolffian duct is the homologue of the tail of the epididymis, the bilobed gland beside it, can be regarded as the seminal vesicle in accordance with its histological structure. (This gland was originally so termed by Pallas (1766) but George and later workers referred to it as the prostate.) This leaves the prostate as disseminated tissue only.

The view of George (1874), expressed later by Oudemans (1892), Weber (1928) and Grassé (1955), that the distribution of accessory sexual organs in the hyrax is special, is thus not upheld, although the bulbo-urethral gland is rather unusual in form. Furthermore, if, as George claimed, the Cowper's gland had previously been considered as the seminal vesicle, much confusion must have originally existed, for even George referred to the seminal vesicle as the prostate gland. The matter could doubtless be firmly settled by chemical means, but the nomenclature presented in this paper is based upon what the authors consider to be fairly substantial histological evidence.

From a functional standpoint, the straight testicular artery and absence of a pampiniform plexus are of interest. These features, together with the origin of the artery from the renal artery, were illustrated by Weber (1928), mentioned by Grassé (1955), and shown again by Harrison (1958). It is evident, therefore, that there is no cooling mechanism for the testes such as is common to most mammals (Harrison & Weiner, 1949). This is perhaps to be expected in view of the abdominal position of the testes in hyrax, and doubtless the direct passage of the artery into the testis parenchyma allows more variation in levels of activity by the testis. The arterial pattern might in part explain the ability of the testes of the hyrax to function at abdominal temperatures, and in this connection, it is interesting that the testicular artery in birds is straight (Glover, 1959), and of even more significance, the testicular artery of the elephant is also straight (Short, Mann & Hayes, 1967).

Maturation of spermatozoa in an intra-abdominal epididymis is very difficult to explain, when in mammals with scrotal testes the process cannot occur normally at abdominal temperature and loss of fertility and ultimate disintegration of spermatozoa ensues when the epididymis is artificially placed in the abdomen (Glover, 1960, 1962; Cummins & Glover, 1968). The preservation of spermatozoa is thus also a problem in the hyrax, but in this regard it is of interest that the main storage centre, referred to here is the tail of the epididymis, lies at the pelvic inlet, and in sexual activity, it may descend well into the pelvis and come close to the perineal surface of the body. Is it possible that the temperature in this part of the body is lower than in the vicinity of the intra-abdominal testes? Measurements of temperature in these different regions of the hyrax are demanded and these could be undertaken in captive animals. Wislocki (1933) carried out many such measurements in a variety of mammals including some testiconda, but the hyrax was not included.

Of probable significance in regard to spermatogenesis and sperm preservation is the fact that hyrax display a considerable degree of thermolability. There are both diurnal and long-term cycles of rectal temperature fluctuation (Sale, in press). These temperature cycles have not yet been fully investigated but there is some evidence suggesting a relationship between long-term fluctuations and sexual activity (Sale, 1965*b*). Might it be that spermatogenesis and sperm storage in the hyrax take place during a period of lowered general body temperature? This seems to be another important question that needs an answer.

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THE PRODUCTION OF SPERMATOZOA IN SOME SPECIES OF 'MAMMALIAN TESTICONDA'

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In most eutherian mammals the testes are situated in a scrotum outside the abdominal cavity, and it has long been realized that in these species spermatogenesis cannot proceed when the testes are in the abdomen. Moreover, it has been shown in rabbits that when the epididymis is secured in the abdomen, the contained spermatozoa do not survive at all well (1, 2). They start to disintegrate after about 7-9 days, and lose their fertilizing capacity much sooner than that (3).

In mammalian testiconda, therefore, in which the testes and their associated ducts are permanently retained in the abdominal cavity, there are two main situations to consider. First, there is the question of how spermatogenesis takes place in these mammals when it is not possible in most other mammals, and secondly, there is the problem of how the spermatozoa survive in an abdominally retained Wolffian duct. It was with these questions in mind that the reproductive organs of the male elephant (Loxondonta), hyrax (Procavia and Heterohyrax), elephant shrew (Elephantulus) and the sloth (Choloepus) were examined.

MATERIALS AND METHODS. Except for the reproductive organs of sloths, which were received from Dr. I. W. Rowlands of London, the material was obtained from East Africa through the courtesy

of the Kenya Game Department. Specimens were fixed in formalin or formal saline (10% formalin in normal saline), and for histological examination, sections were cut at 5 μ and stained with Harris' haematoxylin and eosin or Van Giesen. Examination of the gross structure of the organs was made prior to taking pieces of tissue for sectioning. In all species except the sloth, arteriograms were produced following injection of micropaque (Damancy & Co. Ltd. 100% w/v Barium sulphate) into the aorta. This was done to illustrate the distribution of arterial blood to the testes.

1. DISTRIBUTION OF THE TESTICULAR ARTERY. Arteriograms showed that in all the species under examination the internal spermatic artery is not helical as it is in mammals with scrotal testes. On the contrary, the artery is remarkably straight as it approaches the testis, and it enters the testis parenchyma directly without first taking a superficial course. This can also be said of the artery to the testis of the sloth which was dissected clear of surrounding tissue. A venogram produced from a hyrax confirmed that in this species there is no pampiniform plexus, and it is reasonable to expect this in all the species studied.

2. GROSS AND MICROSCOPIC FEATURES OF THE REPRODUCTIVE ORGANS. There appears to be nothing in the gross structure or histology of the testes in these mammals that gives any explanation of how spermatogenesis proceeds at abdominal temperature. Phases of spermatogenesis can be observed and the interstitium seems to be typical. But the histological features of the Wolffian ducts

and the characteristics of the spermatozoa at different levels of the ducts are interesting in connection with sperm survival.

Hitherto, the part of the Wolffian duct that is not adjacent to the testis in testicond mammals has been referred to as the ductus deferens (4, 5). However, present histological findings indicate that in the elephant, hyrax and elephant shrew this is not so, but that the part of the duct adherent to the testis is homologous to the initial segment and head of the epididymis only. The rest of the duct thus represents the body of the epididymis and the tail of the epididymis, the ductus deferens being very short. That this may be true is confirmed by evidence of spermatozoa undergoing a process of maturation throughout the length of the duct. By contrast, histological evidence from the sloth suggests that in this species the Wolffian duct has become coiled so that the tail of the epididymis lies opposite the inferior pole of the testis. Levels 1, 2 and 3, shown in Fig. 1. represent levels at the initial segment, the head of the epididymis and the tail of the epididymis in that order, and Fig. 1. attempts to illustrate what seem to be comparable levels in the different species.

DISCUSSION. The straight testicular artery demonstrated in these four species of testicond mammals provides a clue as to how spermatogenesis might possibly be maintained at body temperature. It has previously been shown in rats that in experimental cryptorchidism blood flow to the testis increases hardly at all (6). This suggests, since the metabolic demands of the testis might

well be increased under these conditions, that the germinal tissue might thus be deprived of certain essential constituents. Furthermore, it is reasonable speculation that the restriction in blood flow is in some way due to the peculiar vascular architecture of scrotal testes, which includes a pampiniform plexus and a superficial course of the artery on the testis itself. It seems that in testicond mammals no such restriction on the flow of blood would be likely.

As to the Wolffian duct, if the homologies suggested in this paper be accepted, it would mean that in the elephant, hyrax and elephant shrew, mature spermatozoa are stored fairly near to the surface of the body even though the testes are well within the abdominal cavity. In the sloth, the fact that the storage area seems to have moved close to the testis, suggests that perhaps the Edentates illustrate a beginning of testicular descent in the evolution of mammals.

Some of the above mammals have very poor regulation of body temperature, and the importance of this in relation to reproductive activity in the male remains to be investigated.

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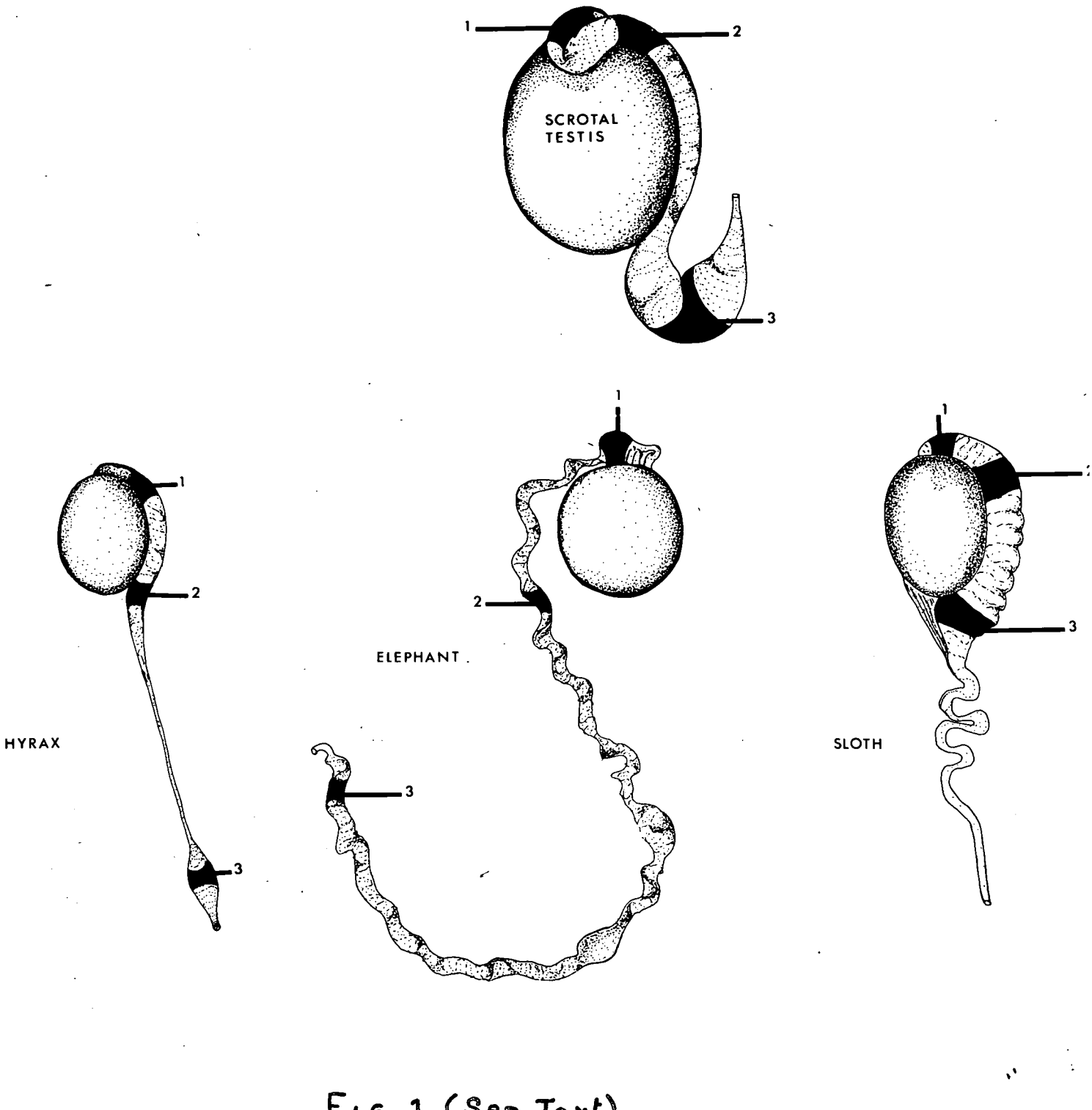

FIG. 1. (See Text)

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SOME ASPECTS OF STRUCTURE AND FUNCTION IN THE MAMMALIAN EPIDIDYMIS

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In the present paper, a further attempt is made to assess possible relationships between structure and function in the epididymis. Of necessity, an appropriate terminology for descriptions of the organ in different species is involved, since the conventional terms of head, body and tail of the epididymis, or caput, corpus and cauda epididymidis, are inadequate and may be confusing. These terms give little indication of variations in microscopic structure and in different species might not even refer to the same region. For instance, Benoit (1926) showed that in the guinea-pig, the part of the epididymis that traverses the surface of the testis and passes over the entire length of the gonad, represents only the initial segment of the duct in terms of histological structure; this is quite different from most other laboratory animals, where the initial segment and head of the epididymis are situated at one pole of the testis only.

Based on histological findings, Nicander (1957a, b) and Reid & Cleland (1957) have demonstrated as many as eight different regions of mammalian epididymides, but for the experimentalist it is laborious and of questionable value to take each one into account repeatedly. What appears to be needed is a fairly simple nomenclature that is rather more precise than the one conventionally used; a nomenclature, perhaps, that might indicate important changes in structure and function along the length of the epididymis but which is simple enough to permit easy description. Before this can be done, however, structure and function in the epididymis needs further analysis, and in the present paper some new approaches to the problem are considered.

EVOLUTION OF THE WOLFFIAN DUCT

An interesting feature of the excurrent duct system of the testes of higher vertebrates is that during the course of evolution its extra-testicular part has been appropriated from the urinary system. Through various intermediate stages, the Wolffian (mesonephric) duct of mammals has come to be used exclusively as an organ of reproduction, contrasting with a theoretical state in primitive gnathostomes where an opisthonephric duct would be expected to convey indiscriminately both gametes and urinary products (Romer, 1960). But the situation is by no means representative of all vertebrates, for in cyclostomes there is no sperm duct and the gametes gain the exterior only after having passed free through the coelomic cavity. In other forms, such as elasmobranchs and

many amphibians, the Wolffian duct is joined caudally by tubules of the kidney, although in some sharks these renal elements have become modified into glandular structures associated, it is believed, with some sort of stimulation of the spermatozoa (Matthews, 1949).

Complete exclusion of renal function from the Wolffian duct provides the most advanced condition, but at one stage in the history of this development there seems to have been an attempt to retrieve the duct for renal function. This is shown by some elasmobranchs where the Wolffian duct is solely associated with the kidney, and the testes have developed their own sperm duct. The condition in Dipnoi is intermediate and only the caudal part of the testis affects a connection with the Wolffian duct by way of the kidney.

SPECIALIZATION OF THE WOLFFIAN DUCT

Acquisition of the Wolffian duct by the testes in higher vertebrates hints that it might not simply be for the conduction of spermatozoa to the urogenital sinus, but that the testis requires it to subserve a more sophisticated function. In this connection it is interesting that the selected duct is hormone-dependent, in contrast to the simple sperm duct of some teleosts (Weisel, 1949). Experiments on the effects of exogenous hormones on the male reproductive tract have been comprehensively reviewed by Dodd (1968), and it is abundantly clear that in most vertebrates the Wolffian duct is dependent upon androgen both for its normal development and adult function. Although information on this in elasmobranchs is evidently scanty, Puckett (1939) and Greenberg (1942) have demonstrated profound responses in amphibian Wolffian ducts to androgens, and the work of Forbes (1941) on the male lizard, *Sceloporus*, gives a clear example of the sensitivity of the reptilian Wolffian duct to injected testosterone. Seasonal changes in the size of the epididymis of the male turtle, *Sternotherus odoratus*, (Risley, 1938) further support this, and presumably a similar situation occurs in passerine birds. Ever since the classical work of Lillie (1916) on sex differentiation and freemartinism, it has been recognized that an androgenic component is needed for the proper development of the Wolffian duct (Jost, 1947, 1951, 1953; Price, 1957; Price & Pannabecker, 1956). Moreover, it is established that, in mammals, proper development of the upper part of the duct demands that there be a connection between the duct and the testis.

When many other specialized features of the epididymis are considered together with this hormone dependence, it is easy to understand why the duct has now long been regarded as more than a simple conducting tube. In mammals there is a characteristic distribution of capillaries to the epididymis (Kormano, 1968), there are specialities in the distribution of blood flow (Setchell, Waites & Till, 1964), the presence of glycerylphosphoryl choline (GPC) in epididymal fluid (Dawson, Mann & White, 1957; Dawson & Rowlands, 1959) and its synthesis in epididymal cells (Wallace, Wales & White, 1966) a high glutamic acid content of epididymal fluid (Setchell, Scott, Voglmayr & Waites, 1969), a progressive increase in post-pubertal content of epididymal sialic acid (Rajalakshmi & Prasad, 1968, 1969) and high concentrations of carnitine (Marquis & Fritz, 1965; Pearson & Tubbs, 1967) not to mention a wide variety of enzymes.

All these features of the epididymis of mammals have recently been excellently reviewed by Hamilton (1971), and Hamilton, Jones & Fawcett (1969) and Hamilton & Fawcett (1970) have indicated that steroidogenesis occurs in the epididymis in rats.

Whilst this high degree of specialization does not justify the opinion of Tournade (1913) and von Lanz (1926) that the secretion of the epididymal cells is important in the maturation of spermatozoa, it does suggest that the epididymis has functions other than the mere conduction of spermatozoa. Moreover, there is more to the physiology of the epididymis than this, for there is morphological evidence that the duct is able to absorb particulate matter (Wagenseil, 1928; Mason & Shaver, 1952; Burgos, 1964; Nicander, 1965) as well as physiological evidence for the absorption of fluid (Crabo, 1965; Waites & Setchell, 1969). Some of these activities, such as phagocytosis of particulate matter, are especially associated with the efferent ductules and the posterior part of the caput epididymidis in rabbits (Nicander, 1963). However, it is difficult to correlate changes in maturing spermatozoa with any specific activity of epididymal cells, largely because the nature of sperm maturation is as yet incompletely understood. Nevertheless, differences in the density of spermatozoa at different levels of the mammalian epididymis can be observed in histological sections and these undoubtedly indicate areas of fluid resorption.

Young (1931) believed that maturation of spermatozoa in the epididymis was a temporal phenomenon, and presumably imagined that variations in cytological structure along the duct were of little importance. But more recent evidence lends support to the contention of Gaddum & Glover (1965) that whilst this is partly true, special activities of the epididymis are also important in sperm maturation. These workers showed, for example, that artificial occlusion of the duct produced unusual behaviour of spermatozoa contained in upper parts of the duct, and Orgebin-Crist (1969) has subsequently shown that the fertilizing capacity of spermatozoa matured in an occluded duct may be less than normal. Glover (1962) showed, however, that the duct epithelium is greatly flattened after occlusion of the epididymis and this has also been confirmed by Bedford (1967) and Orgebin-Crist (1969). Thus, when sperm flow is halted by obstruction of the epididymis the whole character of the duct above the site of obstruction becomes altered and a completely abnormal situation ensues. This must be so whether or not the obstruction is spontaneous or experimentally induced, and hence conditions in a blocked epididymis are not comparable to those prevailing in an intact duct. Successes claimed for the operation of vaso-epididymostomy in man following epididymal block might seem at first sight to run counter to this, but this need not necessarily be so, because there is no evidence to exclude the possibility that patients are invariably sub-fertile after this operation. Also, biological standards of fertility used for man are likely to be quite different from those used for laboratory animals or domestic animals.

It has been reported at this Conference (Calvin & Bedford, 1971) that there are changes in the nucleus of spermatozoa during their maturation in the epididymis, and it is an enlivening thought that different components of sperm maturation might have their own specific requirements. Might it be that maturation in the genome is primarily a temporal matter, while corresponding maturation

changes in the more superficial structures of the spermatozoon are more closely associated with, if not dependent upon, the nature of the milieu? If this were true it would lend significance to the epididymis being both long and structurally complex.

DUAL FUNCTION OF THE EPIDIDYMIS IN RELATION TO ITS GROSS STRUCTURE

Phylogenetically, it seems that the maturation of spermatozoa, begun in the spermatogenic epithelium, has come to be continued and completed outside the testis in the Wolffian duct. In cyclostomes, it is likely to occur within the testes since the gametes are stored there. A large central canal in the testes of some reptiles suggests that this might be so in these species also and no doubt it applies to many amphibians. In more advanced vertebrates, however, the central canal is reduced to a rete testis, and the completion of sperm maturation and the storage of mature gametes have both become extra-testicular events.

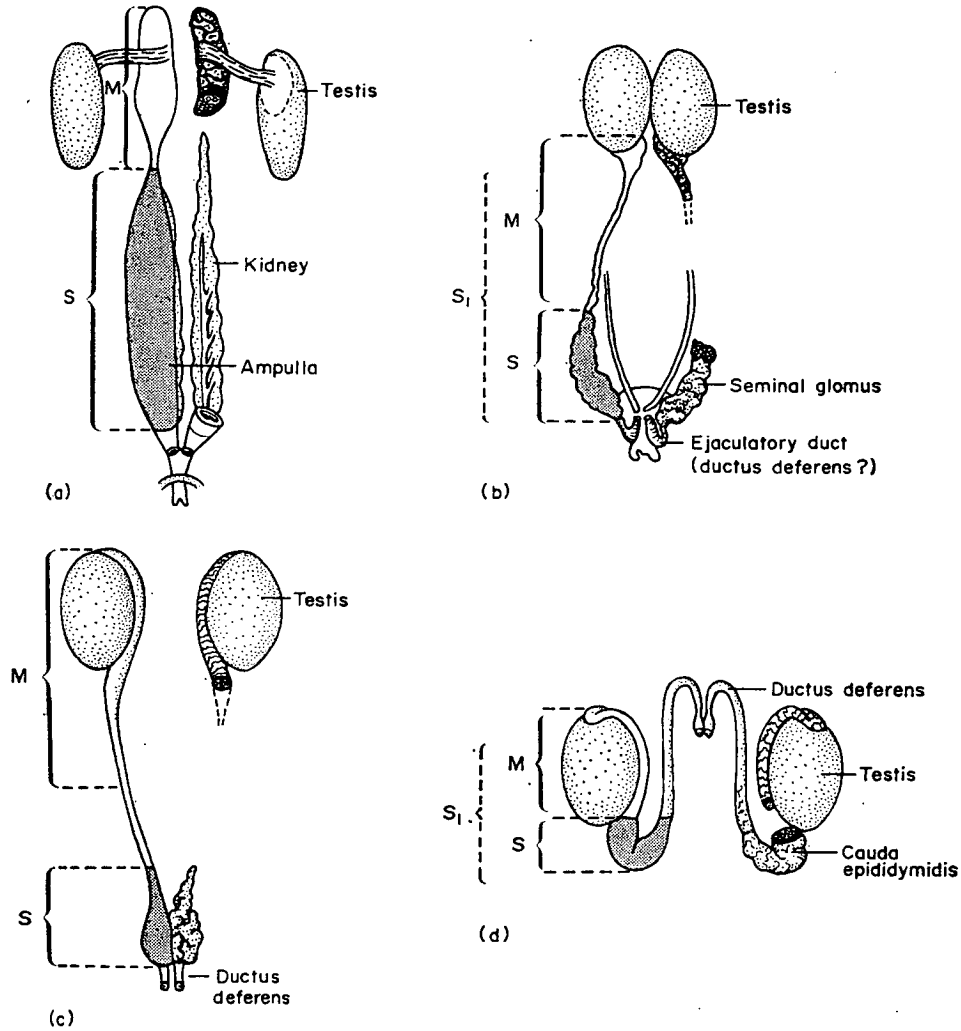
If this extra-testicular maturation of spermatozoa is not a rapid process, it is important that the part of the Wolffian duct where it occurs should be long. It is interesting that, in elasmobranchs such as the basking shark (*Cetorhinus maximus*), excessive coiling of the duct only occurs in its proximal part and that the distal part is straight and expanded to form an ampulla (Text-fig. 1a). Spermatophores are made, and spermatozoa stored, in the ampulla (Matthews, 1949). Thus, it would seem that, in this elasmobranch, maturation of spermatozoa takes place in the upper part of the Wolffian duct and storage of mature spermatozoa is undertaken by the distal part of the duct, which is structurally quite different (see also Text-fig. 1a).

Basically, this dichotomy of both structure and function in the Wolffian duct is also to be found in passerine birds (Marshall, 1961). Here, the 'seminal vesicle' though coiled, is merely a dilation of the Wolffian duct and not an outgrowth of it like a true seminal vesicle. The structure is better referred to as the seminal sac (Marshall, 1961) or seminal glomus (see Witschi, 1955) for it is fundamentally analogous to the ampulla of the Wolffian duct in elasmobranchs in so far as it is for the purpose of sperm storage (Text-fig. 1b).

Up to this point in evolution, therefore, it seems that much of the region of the Wolffian duct where maturation of spermatozoa occurs lies adjacent to the testis (the testis being abdominal), and that the region for the storage of the gametes is remote from the testis and situated nearer the surface of the body. This suggests that a relatively low temperature might be needed for sperm storage, a need that has been proved in mammals with scrotal testes (Glover, 1960) and is strongly indicated in some species of Passeriformes such as the North American swamp sparrow, *Melospiza georgiana* (Wolfson, 1954). In this bird, the seminal sacs descend and come to lie outside the abdomen in a nodular cloacal protruberance which looks rather like a small scrotum.

Spermatozoa are also stored some distance from the gonads in some mammalian species where the testes are abdominal. Such species have been referred to as 'mammalian testiconda' (Owen, 1856; Weber, 1927). Thus, in the rock hyrax (*Procavia* and *Heterohyrax*), the sperm store lies in the pelvic cavity while the testes are situated immediately caudal to the kidneys (Text-fig. 1c). Glover & Sale

(1968) have suggested that, in this species, the sperm store is both homologous and analogous to the cauda epididymidis of mammals with scrotal testes, since spermatozoa taken from the region are actively motile *in vitro* and so contrast



TEXT-FIG. 1. Diagrams to illustrate suggested homologous and analogous areas of the male reproductive tract (Wolffian duct) in a number of vertebrate types. In each diagram, comparable regions are illustrated on the left, and the sperm store is shaded in each case, whilst on the right of each diagram, the outer appearance of the tract is illustrated with the Wolffian duct sectioned at its extremity. M = part of Wolffian duct where spermatozoa undergo maturation. S = part of Wolffian duct where mature spermatozoa are stored. As suggested in text, sperm store, seminal glomus of birds and terminal segment of mammals all refer to the same area of the Wolffian duct but, as seen in the rabbit (Text-fig. 2a), this may not always be represented by the cauda epididymidis alone.

(a) Testes and adult Wolffian duct of an elasmobranch fish, the basking shark, *Cetorhinus maximus* (modified from Matthews, 1949). (b) Testes and adult Wolffian duct of the bird (modified from Witschi, 1955). S = sperm store in passerine birds, S_1 = suggested extent of sperm store in non-passerine birds like the domestic cock, *Gallus domesticus* (see text). (c) Testes and adult Wolffian duct of the rock hyrax, *Procavia capensis*. True extent of sperm store (terminal segment) as yet unknown (see text). (d) Testes and adult Wolffian duct of typical mammal with scrotal testes. S_1 = extent of sperm store (terminal segment) in domestic rabbit, *Oryctolagus cuniculus*.

with those taken from the testis, or that part of the epididymis adjacent to the testis, where the capacity for motility *in vitro* is minimal. Subsequent work (Glover, in preparation) has shown, on the grounds of sperm morphology, that this interpretation is probably correct and that it might apply to a variety of testicond mammals, including the elephant, *Loxodonta africana*, the North African elephant shrew, *Elephantulus rufescens* (*rozeti*), and the four-toed and forest elephant shrews, *Petrodromus tetradactylus* and *Petrodromus sultan*.

Munro (1938) has shown in the domestic cock that spermatozoa taken from the lower part of the Wolffian duct are much better able to fertilize than those taken from the testis or from the duct at the level of the testis. There is no seminal sac in these birds and it rather looks as if, in contrast to passerine birds, virtually the entire duct distal to the testis represents the sperm store or cauda epididymidis. This basically supports the view of Lake (1957).

All this morphological evidence strongly indicates that, in birds and testicond mammals, the part of the Wolffian duct adjacent to the testis represents only the initial segment and head of the epididymis, and that the term 'vas deferens' universally used for the rest of the duct is a misnomer. It seems likely, in fact, that the true ductus deferens in these species is very short (Text-fig. 1).

If this interpretation is accepted, it is easy to recognize that, with the descent of testes into a scrotum, the sperm store came to lie progressively nearer to the testis, and the ductus deferens lengthened correspondingly (Text-fig. 1d). Any alternative explanation of the history of the duct demands that a scrotal position for the testis is the more primitive condition, and this seems unlikely.

If the basic concept of the epididymis having two distinct parts, each serving a separate function, be applied to mammals generally, it would be reasonable to anticipate that the initial segment and head of the epididymis are concerned with sperm maturation and that the tail of the epididymis is associated with the storage of mature spermatozoa. Broadly, this would appear to be true, and the body of the epididymis, although some spermatozoa complete their maturation in it (Bedford, 1966; Orgebin-Crist, 1967), might be regarded as essentially a connection or link between the head of the epididymis and the sperm store.

EXPLANATION OF PLATE 1

Histological sections and electron micrographs of the three suggested segments of the epididymis of the golden hamster, *Mesocricetus auratus*, used to typify the main structural features as seen in all the mammalian species mentioned.

FIG. 1. Histological section of initial segment. H & E, ×180.

FIG. 2. Section of the middle segment (see text and Fig. 7). H & E, ×180.

FIG. 3. Section of middle segment under higher magnification to illustrate supranuclear vacuoles more clearly. H & E, ×450.

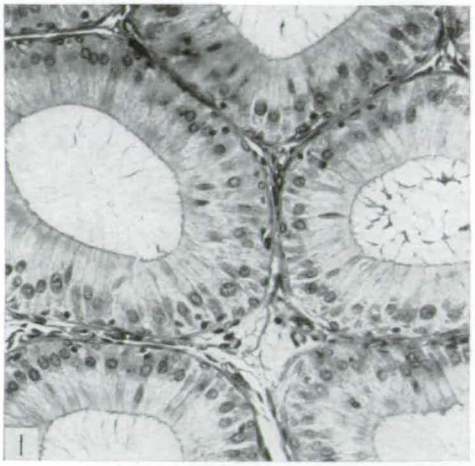
FIG. 4. Section of terminal segment. H & E, ×180.

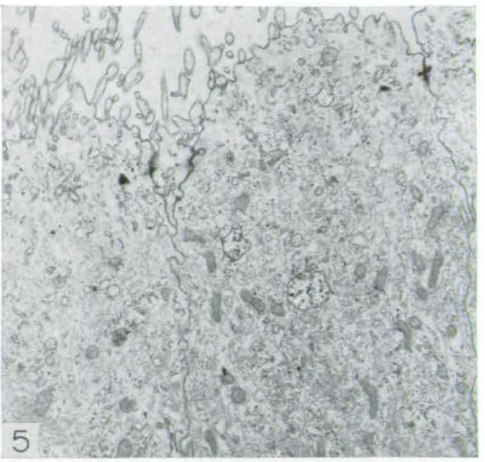
FIG. 5. Electron micrograph of initial segment showing micropinocytic vesicles at the surface and several smallish multivesicular bodies. Osmium fixation and lead citrate and uranyl acetate staining, ×4000.

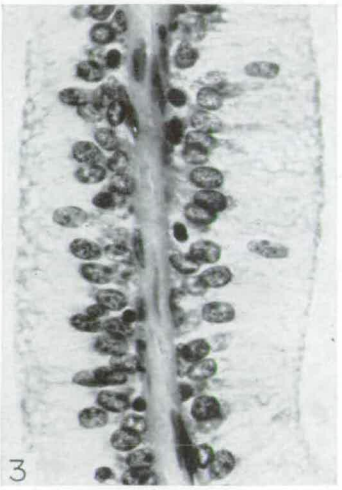
FIG. 6. Electron micrograph of middle segment showing 'macropinocytosis' and large multivesicular bodies. This represents the first part of the middle segment (see text). Same fixation and staining as above.

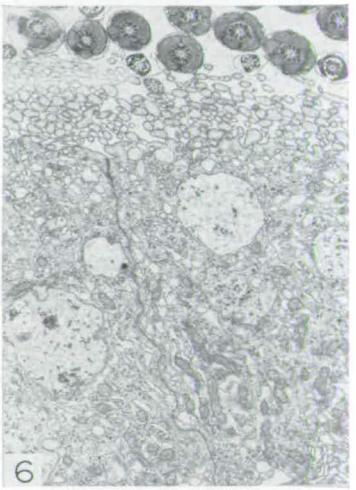
FIG. 7. Electron micrograph of terminal segment showing micropinocytic vesicles and one multivesicular body on the right. Same fixation and staining as above, ×4000.

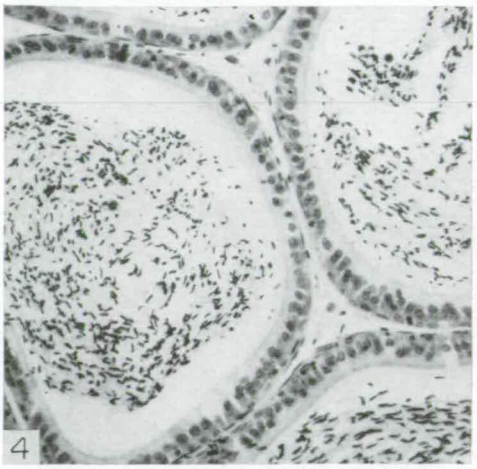
PLATE 1

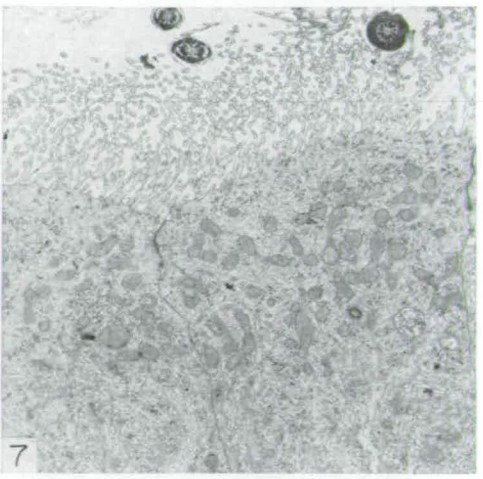










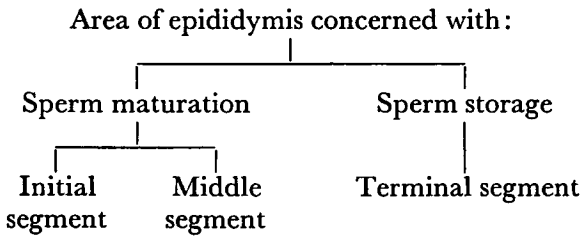


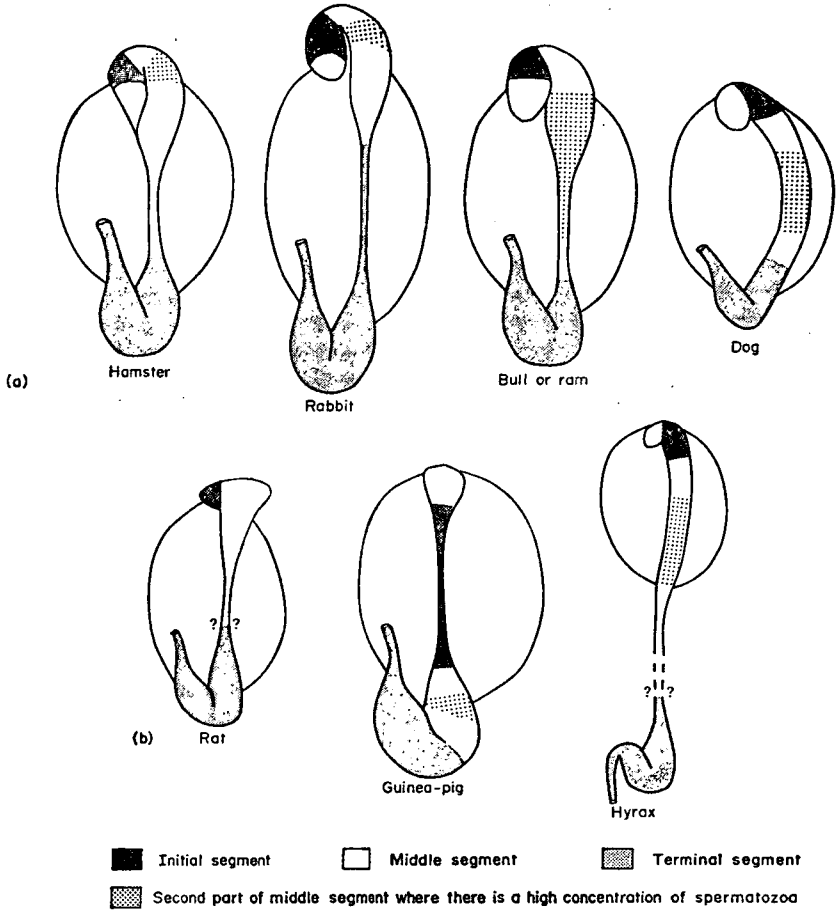
(*Facing p.* 44)

In many higher vertebrates, a simple division of the epididymis into two parts is less easily recognized than in an elasmobranch, because the sperm store is coiled like the rest of the duct. Moreover, in mammals, the microscopic structure of the epididymis changes along its length (Hammar, 1897; Nicander, 1957a, b; Reid & Cleland, 1957) and owing to slight differences in the way it has evolved, the degree of coiling and the position of the coils in relation to the testis is by no means constant between species. A further complication arises from the fact that, whilst the histology and cytology of the initial segment and also that of the tail of the epididymis are relatively straightforward and largely independent of species, the intervening part of the duct appears to vary somewhat in different species both in its detailed microscopical structure and length.

A STANDARD FOR DESCRIPTION OF MAMMALIAN EPIDIDYMIDES

In spite of these difficulties it seems feasible to divide most mammalian epididymides, on the basis of histological and cytological characteristics, into an 'initial' segment, a 'middle' or 'intermediate' segment and a 'terminal' segment. The first two of these might be regarded together as making up that part of the epididymis concerned with sperm maturation, and the terminal segment, as being the sperm store (see below). These segments do not correspond to the areas described as head, body and tail of the epididymis, for these are terms that relate only to macroscopic structure and are used simply for convenience in general orientation; they are often entirely unhelpful as far as the microscopic picture is concerned. Based on histological and fine structure, division of the epididymis into three segments only both simplifies earlier descriptions and affords some rationality to the structure of the tract by relating it to its function and evolution. But how widely can it be applied to the epididymis of different species? Evidence to date shows that the principle can best be applied to the epididymis of the dog, the hamster and the rabbit among the domesticated and laboratory animals (Text-fig. 2) and although the situation in the rat and mouse is a little less certain it is probable that the epididymis of testicond mammals can also be described in these terms (Text-fig. 2). The ductus deferens in every species will contain spermatozoa, but these may be taken as the spermatozoa that have spilled over from the main sperm store. It might, in fact, be argued that the terminal segment in mammals should not strictly be referred to as a sperm store, since it appears as a continuation of the middle segment and spermatozoa continuously pass through it. But this is not really a tenable argument for, although the segment might have become slightly changed in nature during the course of its evolution, it remains fundamentally a sperm store.





TEXT-FIG. 2. Diagrams to illustrate the epididymis in a variety of mammalian species when divided into the three segments, initial, middle and terminal, based on histological and cytological features (see text). It will be noted that the line of demarcation between the middle segment and the terminal segment in the epididymis of the rat and rock hyrax is as yet uncertain.

Histological basis for description

Initial segment. This first part of the epididymis is generally characterized by high epithelium which has extremely long straight stereocilia which almost obliterate the lumen (Benoit, 1926). Usually very few spermatozoa can be seen in histological sections from this region (Pl. 1, Fig. 1).

Middle segment. Here, stereocilia occur regularly and unlike those in the initial segment, they are usually seen to be bent. The lumen of the duct is wider in this region, although it varies in degree at different levels. Supranuclear vacuoles in the epithelium are particularly characteristic of the segment, although again the frequency and extent of their occurrence might differ from species to species (Pl. 1, Figs. 2 and 3).

Terminal segment. The epithelium of this segment is usually seen to be lower than

in other regions, the stereocilia are low and thin, and the lumen, which is normally packed with spermatozoa, is very wide (Pl. 1, Fig. 4). This is less obvious if there is *post mortem* contraction of the smooth muscle in the duct or if, for some reason, the segment is previously emptied of spermatozoa. In the second instance, pressure is relieved and the epithelium looks much higher.

Cytological features

Initial segment. Evidence of micropinocytosis is invariably present in sections examined under the electron microscope, but it is extremely variable in extent both within and between species. Apical multivesicular bodies are common, however, and there can be little doubt that some absorption in this area is possible. These cytological characteristics are illustrated in Pl. 1, Fig. 5.

Middle segment. In this region pinocytosis is much more conspicuous and the supranuclear vacuoles are quite striking when seen in electron micrographs. It is of interest that, when they occur as circumscribed dense bodies, they provide an area of concentrated acid phosphatase activity. The distribution of these vacuoles throughout the area is also interesting, for their concentration in the cells differs in different regions of the segment. In the first part of the segment the vacuoles are particularly profuse (Pl. 1, Fig. 6), but lower down they become more sparsely populated before a final region of the segment where the cells are again quite heavily vacuolated. Obvious crowding of the spermatozoa in the lumen occurs in the second part of this segment, indicating massive resorption of fluid in the first part. This fits in well with the cytological picture of excessive vacuolation in the region.

Terminal segment. From electron micrographs of this segment there is little evidence of extensive absorption. The small apical vesicles can only be described as micropinocytic and the multivesicular bodies that are present are only small and fairly sparse (Pl. 1, Fig. 7).

Using these criteria for recognition of the three segments, it is apparent from the resulting diagrams in Text-fig. 2 that there are unquestionable differences in the position and extent of the different segments in different species of mammals. This is particularly well shown in comparing the epididymis of the rabbit, for instance, with that of the guinea-pig (Text-fig. 1). The epididymis of the rat has a distinctive microscopic structure, while the ram, bull, dog and hamster bear a closer resemblance to each other in this respect. When this classification is applied to the epididymis of the hyrax, it confirms the evolutionary trends outlined earlier.

CONCLUSIONS

It is well known that, in mammals, spermatozoa complete their maturation in the epididymis and that they are stored there before ejaculation. It is rarely emphasized, however, that these events take place in different parts of the duct and probably require quite different environmental conditions. This is important because, in spite of the popular view that ageing in spermatozoa is merely a continuation of the maturation process, it may be that sperm storage is a distinct physiological entity and that the sperm store might be functionally quite separate from other parts of the duct. In this paper, evidence is produced from testicond

mammals and some lower vertebrates to support this possibility, and the sperm store is referred to as the terminal segment of the epididymis in order to distinguish it from the initial segment and middle segment, which would appear to be more concerned with sperm maturation. It is suggested that a simplified nomenclature of this kind might perhaps be applicable to the epididymis generally, though it must be made clear that variations in detailed structure are to be expected in different species. Moreover, the picture will also vary according to the nature and amount of luminal contents, especially in upper regions of the duct, and it can thus be changed by experimental interference such as, for example, artificial occlusion of the duct or experimental cryptorchidism. In addition, the histological and cytological features of the epididymis are greatly modified by changes in the hormonal climate, so that sections taken from sexually active animals look totally different from those obtained from sexually quiescent ones of the same species.

However, with these reservations, the authors believe that it is reasonable to describe the mammalian epididymis as consisting of three basic segments, and that in relation to physiological activity it might be prudent to consider the terminal segment as distinct and separate from the rest of the duct.

ACKNOWLEDGMENTS

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Comparative histochemical localization of lysosomal enzymes in mammalian epididymides

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INTRODUCTION

The epididymis is considered by some authors to be mainly an absorptive organ. Vital dyes (Trypan blue and India ink) have been shown to be absorbed from the luminal contents of the proximal part of the duct (Mason & Shaver, 1952; Shaver, 1954) and more recently the phenomenon of absorption has been studied by electron microscopy (Nicander, 1965; Burgos, 1964, 1967; Sedar, 1966; Friend & Farquhar, 1967). However, the relative importance of different regions of the epididymis in relation to absorption is not yet clear, although studies carried out by Shaver (1954), Nicander (1965) and Burgos (1964, 1967) suggest that the proximal duct has an important role in the absorption of some constituents of fluid emanating from the testis. The cauda epididymidis and the ductus deferens are considered by others to be important in the resorption of non-ejaculated spermatozoa (Simeone & Young, 1931; Grant, 1958; Amann & Almquist, 1962; Niemi, 1965; Barker & Amann, 1969).

The lysosomes, which contain several acid hydrolases, constitute an intracellular digestive system (de Duve, 1959, 1963), and have been implicated in the digestion of ingested substances in various types of mammalian cell (Straus, 1967). There has been little evidence, however, that in the epididymis hydrolytic enzymes are localized in the lysosomes. The occurrence of acid phosphatase has been reported in the epididymis of the rat (Martan & Risley, 1963), mouse (Allen & Slater, 1958; Martan & Allen, 1964), rabbit (Linnetz & Amann, 1968), bull (Rollinson, 1955; Roussel & Stallcup, 1967) and man (Montagna, 1952) and non-specific esterase has been examined in the mouse epididymis (Allen & Slater, 1957). No other studies of a complete nature have been carried out on the distribution of non-specific esterase in other mammalian epididymides, although several incidental observations appear in the literature (Nachlas & Seligman, 1949; Chessick, 1953; Mazaty & Bourne, 1955). The histochemical localization of β-glucuronidase in mammalian epididymides has received little attention, although Hayashi (1964, 1967) has a brief report on its distribution in the rat epididymis. Studies on acid phosphatase and non-specific esterase have been carried out on unfixed or acetone-fixed material in which rupture of the lysosomes and considerable diffusion may have occurred (Holt & Hicks, 1964).

Improved histochemical methods for several hydrolytic enzymes are now available and in the present study the distribution of three hydrolytic enzymes (acid phos-

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phatase, β-glucuronidase and non-specific esterase) was examined in the epididymides of four different species of mammal, namely, the ram, rabbit, rat and hamster. The aim was to determine whether or not these enzymes occurred mainly in the lysosomes of the epithelial cells, and to study their precise distribution in different regions of mammalian epididymides.

MATERIALS AND METHODS

Light microscopy

Three adult rams were examined and also four rabbits, six rats and six hamsters. Material from rams was obtained from the slaughter house, but epididymides from rabbits, rats and hamsters were removed from animals anaesthetized with sodium pentobarbitone (Nembutal, Abbott Laboratories). Small slices of epididymal tissue about 2 mm thick were cut from different levels of the duct (Fig. 1), fixed immediately in 2·5% glutaraldehyde and 0·1 M phosphate buffer at pH 7·4 (Sabatini, Bensch & Barrnett, 1963), and held at 0–4 °C for 2–3 hours. Similar tissues were collected from the rams and fixed within about 30 minutes of death of the animal. The fixed tissues were washed overnight in 0·2 M cacodylate buffer containing 0·2 M sucrose at 0–4 °C and then frozen in isopentane cooled with dry ice. Sections were cut at 5 μm in a cryostat at −20 °C and picked on to coverslips coated with a thin layer of gelatin.

Acid phosphatase was demonstrated by the modified technique of Gomori (Pearse, 1960) at pH 5·2 using sodium β-glycerophosphate as substrate. β-glucuronidase was demonstrated as described by Hayashi, Nakajima & Fishman (1964) using naphthol AS-B1-β-glucuronide (Sigma) as substrate at pH 5·2, and non-specific esterase (Pearse, 1960) using sodium α-naphthyl acetate at pH 7·4. Sections were incubated for 5, 10, 15 and 20 minutes at 37 °C for acid phosphatase, 20, 30 and 40 minutes at 37 °C for β-glucuronidase and 5 and 10 minutes at room temperature for non-specific esterase. Substrates were prepared immediately before use and fresh staining solution was used for each batch of sections. Controls were incubated in substrate-deficient media.

Electron microscopy

To study the ultrastructural localization of acid phosphatase, material from two hamsters was prepared for electron microscopy. Small pieces, measuring about 1 × 2 × 2 mm, from different regions of the epididymis were fixed in glutaraldehyde and washed in cacodylate buffer in the same way as described for light microscopy. The washed pieces were incubated for 20 minutes in the modified Gomori's substrate, rinsed in distilled water and post-fixed in 1% phosphate buffered osmium tetroxide (Millonig, 1961). After rapid dehydration in acetone the pieces were embedded in Epon (Luft, 1961). Thick sections, about 1 μm, were cut with an LKB Ultratome and examined under a phase contrast microscope before cutting thin sections from the surface of the block. Epon sections were placed on uncoated copper grids and examined in a JEM7A electron microscope, before and after staining with uranyl acetate (Watson, 1958) and lead nitrate (Venable & Coggeshall, 1965). Controls were incubated in substrate-deficient media. Some sections were also prepared from similar regions for normal fine structure.

RESULTS

The results are summarized in Fig. 2.

Both acid phosphatase and β-glucuronidase were demonstrated as discrete granules in the lining epithelium of the epididymides of all the species studied. No reaction was seen in the general cytoplasm, the nuclei or in the stereocilia of these cells. Non-specific esterase, however, was demonstrated both in the form of granules and in the general cytoplasm. In the ram and hamster the reaction for non-specific esterase was mainly in the form of granules, whilst in the rabbit and rat it was mainly cytoplasmic. The intensity and number of granules stained for each of the three enzymes were different in separate regions of the epididymis and varied from species to species: for purposes of description, the terminology put forward by Glover & Nicander (1971) (dividing the epididymis into initial, middle and terminal segments) is used (Fig. 1).

Ram

Acid phosphatase

The columnar cells of the middle segment had the highest acid phosphatase activity (Fig. 4). Numerous granules were seen in the general cytoplasm, with a greater concentration towards the basal half of the cells. The initial segment showed few granules, and these were mostly confined to the supranuclear region (Fig. 3). In the terminal segment and the ductus deferens granules were concentrated in the basal region of the columnar cells (Fig. 5), but these were less numerous in the ductus deferens.

β-glucuronidase

The granules stained for β-glucuronidase were localized at the same sites as acid phosphatase and non-specific esterase (compare Figs. 5, 7, 8). However, the intensity of the enzyme varied at different levels of the epididymis. Activity was relatively high in the terminal segment (Fig. 8), moderate in the middle segment, and low in the initial segment. In the connective tissue and in the luminal contents, a few cells resembling macrophages showed a positive reaction for this enzyme.

Non-specific esterase

The distribution of this enzyme was both granular and cytoplasmic. The efferent ductules showed a diffuse moderate reaction in the general cytoplasm, whilst the initial segment showed a weak diffuse reaction, with a few granules in the supranuclear region of the columnar cells. The middle segment showed the highest activity, and here numerous granules were seen, with a high concentration of the enzyme in the basal half of the columnar cells (Fig. 6). In the terminal segment, the granules were almost entirely confined to this part of the columnar cells (Fig. 7). The muscular coat of the ductus deferens showed a strong diffuse reaction for this enzyme.

Rabbit

Acid phosphatase

The columnar cells of the middle segment had the highest activity of acid phosphatase (Fig. 9), and some granules were already seen after five minutes of incubation. The granules were concentrated in the basal half of the cells, both below and

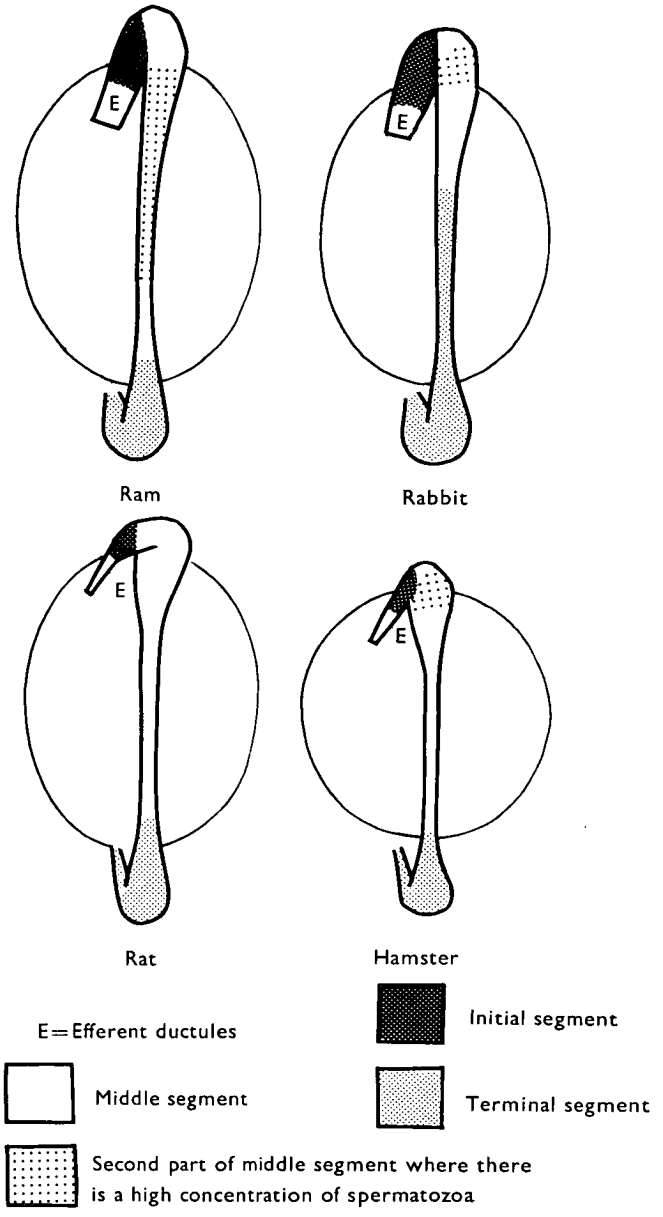


Fig. 1. Schematic drawings of the testes and epididymides of ram, rabbit, rat and hamster showing the three segments, initial, middle and terminal, according to the classification of Glover & Nicander (1971).

above the nucleus. By contrast, the reaction in the initial segment was only moderate. The reaction decreased gradually as it was traced distally towards the terminal segment (Fig. 10), where it was weak to moderate. Activity in the initial and terminal segments was concentrated above the nucleus.

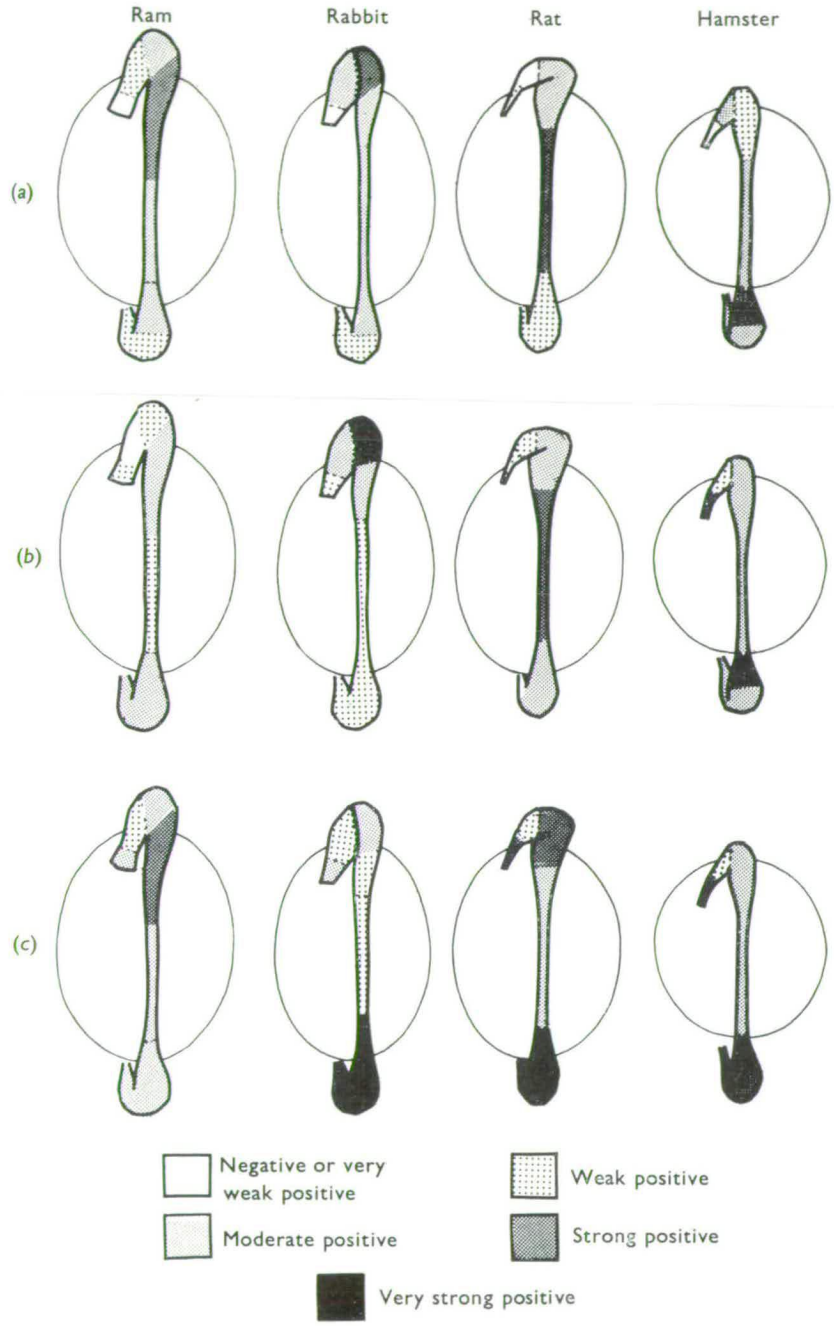
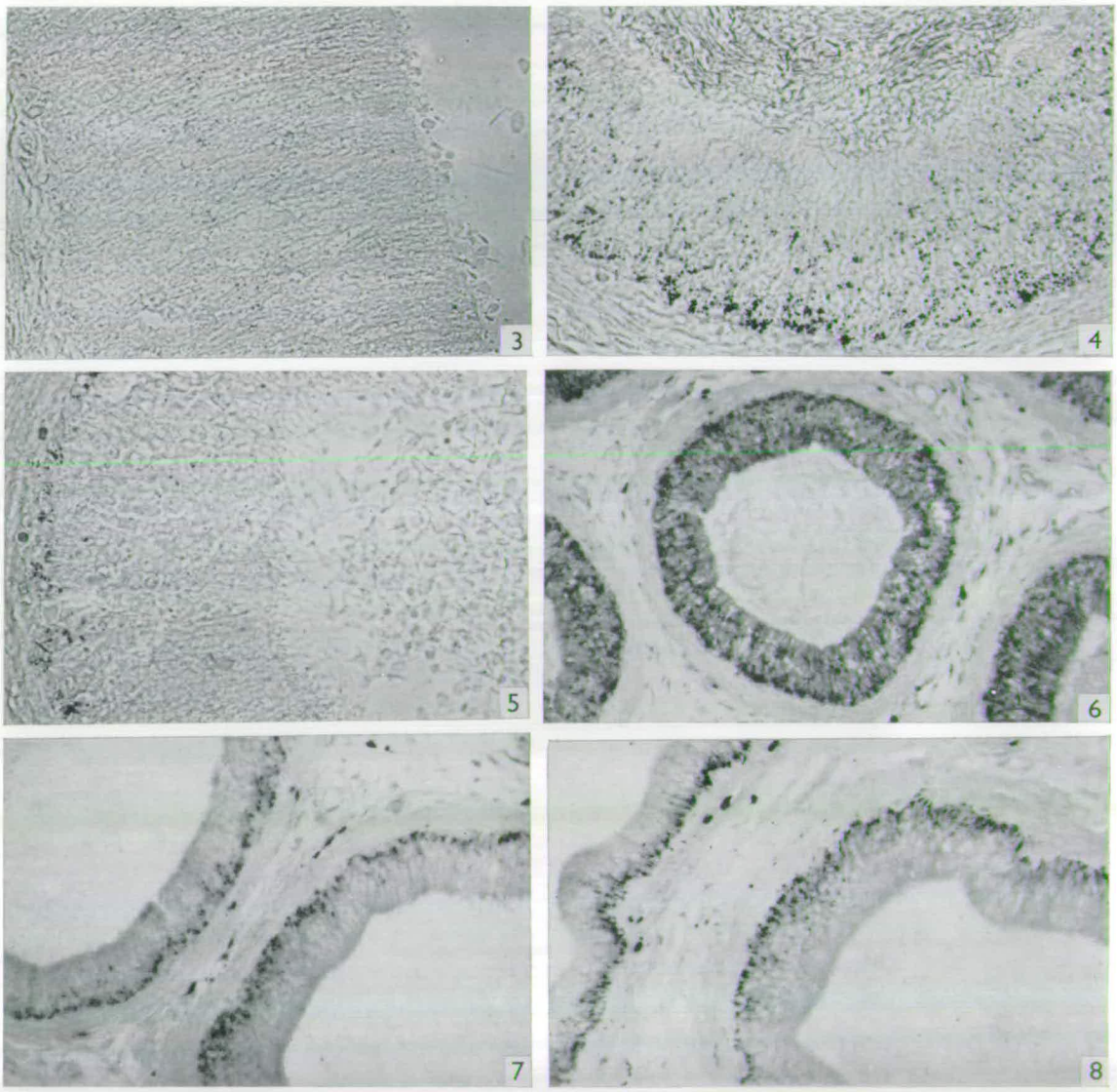


Fig. 2. Schematic drawings of the testes and epididymides of ram, rabbit, rat and hamster: (*a*) showing the distribution of acid phosphatase in the epididymides of the four species: (*b*) showing the distribution of β-glucuronidase in the epididymides of the four species; and (*c*) showing the distribution of non-specific esterase in the epididymides of the four species.



Figs. 3–5. Ram epididymis. Acid phosphatase, lead nitrate method, incubation time 10 min. × 750.

Fig. 3. Initial segment.

Fig. 4. Central part of the middle segment.

Fig. 5. Proximal part of the terminal segment.

Figs. 6, 7. Ram epididymis. Non-specific esterase, α-naphthyl method, incubation time 10 minutes. × 250.

Fig. 6. Central part of the middle segment. The few dark particles in the intertubular connective tissue are pigment cells.

Fig. 7. Proximal part of the terminal segment.

Fig. 8. Ram epididymis. Proximal part of the terminal segment. β-glucuronidase, hexagonium pararosanilin method, incubation time 30 minutes. × 250.

β-glucuronidase

The intensity and localization of this enzyme followed almost the same pattern as that described for acid phosphatase. The highest activity (Fig. 12) was seen in the middle segment (this was characteristic of all four species examined) whilst the reaction was only moderate in the initial segment (Fig. 11) and the distal half of the middle segment. Proceeding distally from the lower part of the middle segment (Fig. 13), the reaction decreased gradually towards the terminal segment, where it was weak. A few cells in the intertubular connective tissue and in the luminal contents (Fig. 11) showed a positive reaction for this enzyme.

Non-specific esterease

The reaction was mainly cytoplasmic throughout the epididymis. The efferent ductules showed a moderate reaction and the intensity decreased to a low level in the initial segment. In the middle segment there was a moderate diffuse reaction in the columnar cells, with higher activity towards the stereocilia and the apical cytoplasm (Fig. 14). There was an intense diffuse reaction in the distal part of the terminal segment.

Rat

Acid phosphatase

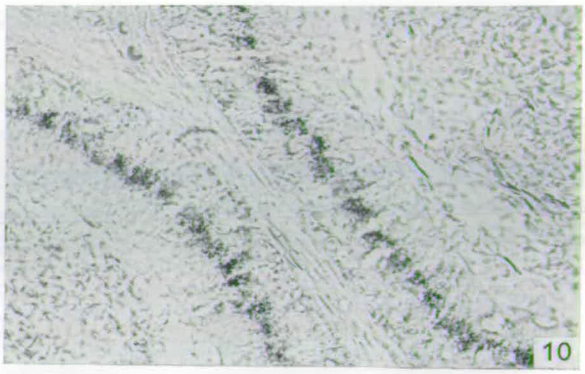
The activity was localized in the supranuclear region throughout the epididymis, and the granules were larger than in the previous two species. The efferent ductules and the initial segment revealed low activity and a few granules were seen only after long incubation periods (25–30 minutes). Proceeding distally, the activity gradually increased towards the distal part of the middle segment (Figs. 15, 16), which showed the highest activity. In the terminal segment (Fig. 17) the activity dropped to a low level, and twice the time of incubation was needed to give a comparable result to that found in the middle segment. The terminal segment was characterized by the presence of numerous columnar cells, which were packed with acid phosphatase positive granules. These cells were rare in the initial and middle segments.

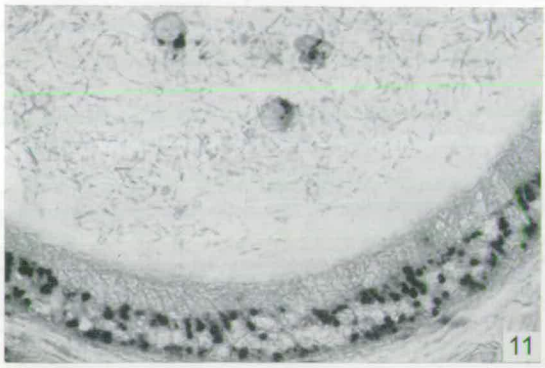
β-glucuronidase

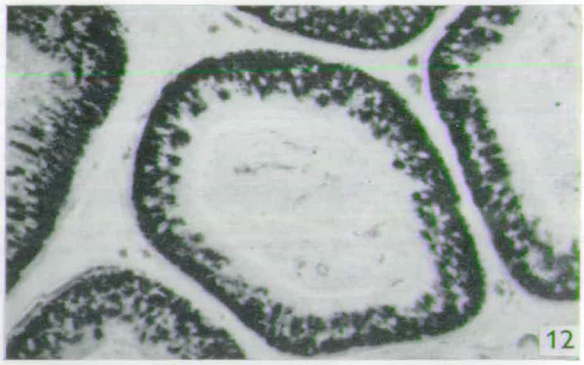
The intensity and localization of β-glucuronidase were similar to acid phosphatase. The activity was low in the initial segment, but increased in the middle segment (Fig. 18) and then decreased in the terminal segment (Fig. 19). The intertubular connective tissue contained numerous cells stained for this enzyme. A few cells in the luminal contents were also stained.

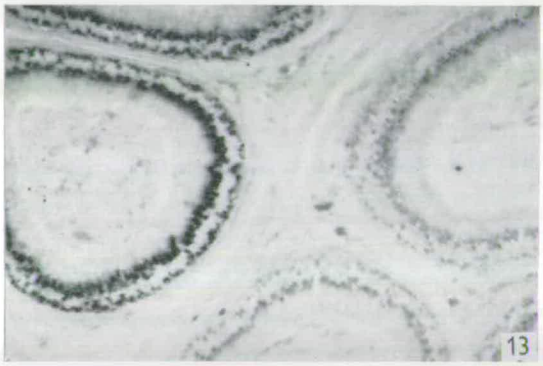
Non-specific esterase

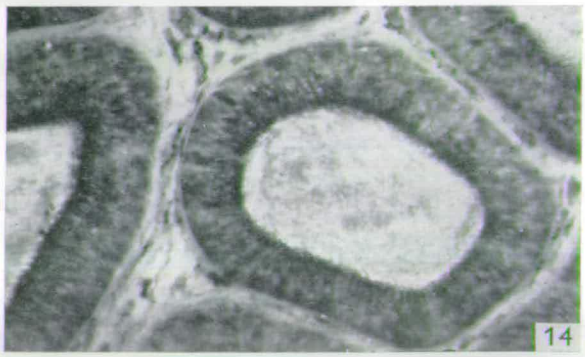
The reaction was mainly cytoplasmic, except in the efferent ductules and the proximal part of the middle segment, where numerous granules were seen. The initial segment showed a diffuse weak reaction in the columnar cells, with higher activity in the basal cells. A few cells showed higher activity in their apical cytoplasm, and these cells were more numerous towards the proximal part of the middle segment. In the middle segment the activity was low to moderate, but increased to a high level in the terminal segment (Fig. 20).











Figs. 9, 10. Rabbit epididymis. Acid phosphatase, lead nitrate method, incubation time 10 minutes. × 750.

Fig. 9. Proximal part of the middle segment.

Fig. 10. Central part of the terminal segment.

Figs. 11–13. Rabbit epididymis. β-glucuronidase, hexagonium pararosanilin method, incubation time 30 minutes.

Fig. 11. The distal part of the initial segment. Note the presence of some cells in the lumen showing positive reaction for this enzyme. × 750.

Fig. 12. The proximal part of the middle segment. × 250.

Fig. 13. The distal part of the middle segment. Note the decrease of activity as one descends down the epididymis. × 250.

Fig. 14. Rabbit epididymis. Proximal part of the middle segment. Non-specific esterase, α-naphthyl method, incubation time 10 minutes. × 250.

Hamster

Acid phosphatase

(*a*) *Light microscopy*. Activity was typically located above the nucleus. It was maximal in the proximal part of the terminal segment (Fig. 23) where, besides the supranuclear activity, a few granules were seen in the perinuclear region of the columnar cells. The activity in the initial segment was moderate (Fig. 21), but lower in the proximal part of the middle segment (Fig. 22). The distal part of the middle segment and the terminal segment showed only a moderate reaction. A number of cells, scattered between the columnar cells of the terminal segment, had a very intense acid phosphatase activity, but such densely stained cells were rare in the initial and middle segments.

(*b*) *Electron microscopy*. Throughout the epididymis the reaction product was found in decreasing concentration in dense bodies, multivesicular bodies and in some Golgi vesicles but the Golgi cisternae were non-reactive (Figs. 27, 28). The reactive dense bodies and multivesicular bodies were mainly present in the region between the nucleus and the Golgi apparatus. A few reactive dense bodies were sometimes seen in the infranuclear region, but no precipitate was seen in the apical region of the columnar cells.

β-glucuronidase

The localization of this enzyme was similar to that of acid phosphatase. The efferent ductules showed a strong reaction, which reached a low level in the initial segment. The middle and terminal segments showed a moderate reaction, but slightly higher activity occurred towards the proximal part of the terminal segment (Figs. 24, 25).

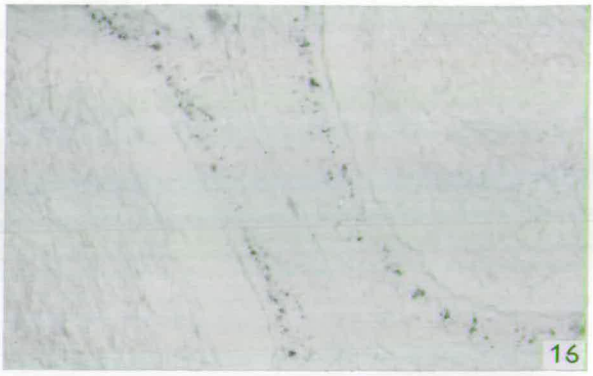
Non-specific esterase

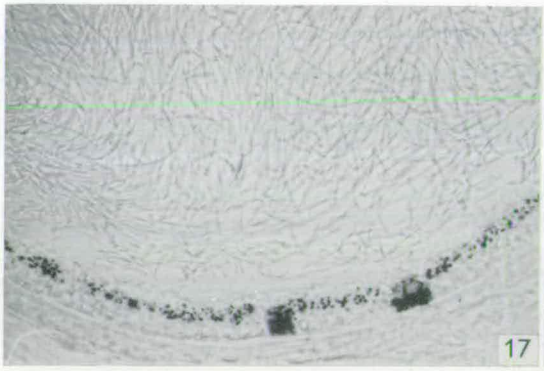
The reaction was both granular and cytoplasmic (Fig. 26), and the localization and intensity of stained granules were similar to those of the previous two enzymes.

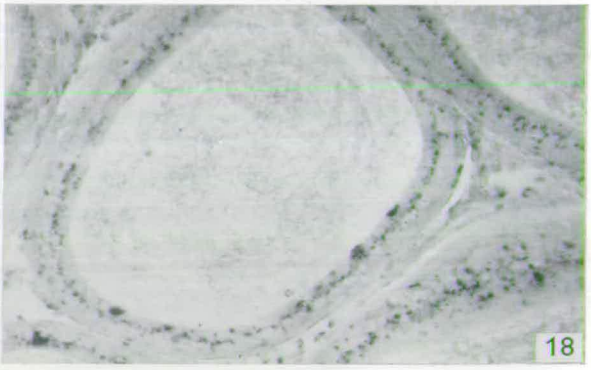
DISCUSSION

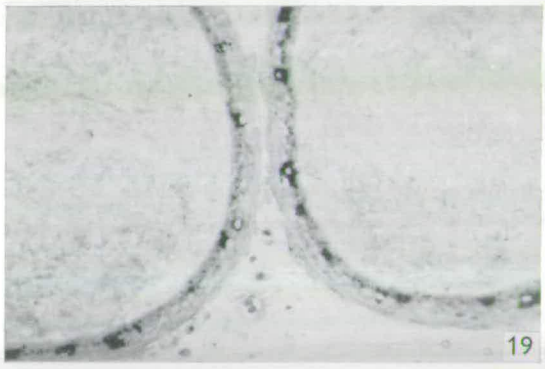
In this study acid phosphatase was always confined to discrete granules in specific parts of the epithelial cells, and there was no diffuse reaction in the general cytoplasm, nuclei or stereocilia. This finding contrasts with most previous studies on the distribution of the enzyme in mammalian epididymides, where a diffuse reaction in all regions of the epithelial cells has invariably been reported. However, most earlier work has been carried out on unfixed or acetone-fixed material where long incubation periods have been used, and the diffuse reaction might thus well have been an artifact.

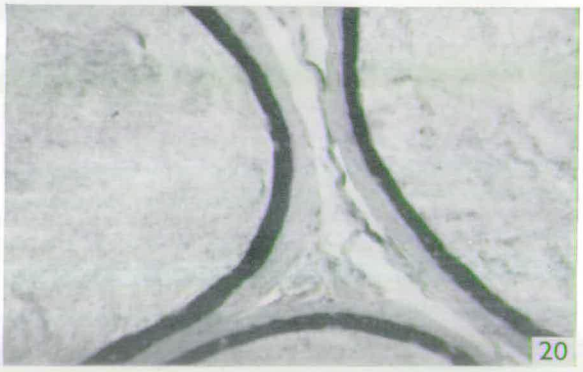
In the present study, β-glucuronidase was also localized as discrete granules, but non-specific esterase was demonstrated both in the general cytoplasm and in the form of granules. Granules stained for each enzyme appeared to be localized at similar sites, which strongly suggests that the sites represented lysosomes. This was confirmed by electron microscopy.











Figs. 15–17. Rat epididymis. Acid phosphatase, lead nitrate method. × 750.

Fig. 15. Proximal part of the middle segment. Incubation time 10 minutes.

Fig. 16. Distal part of the middle segment. Incubation time 10 minutes.

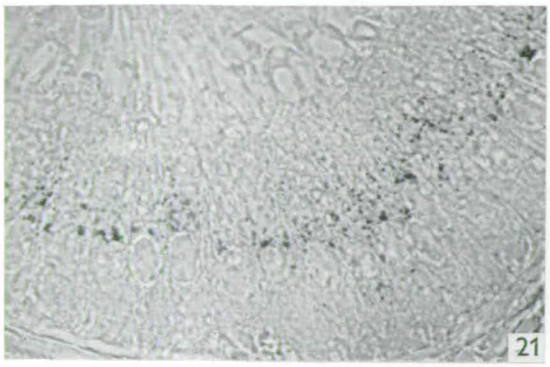
Fig. 17. The terminal segment. A few cells are packed with acid phosphatase positive granules. Incubation time 20 minutes.

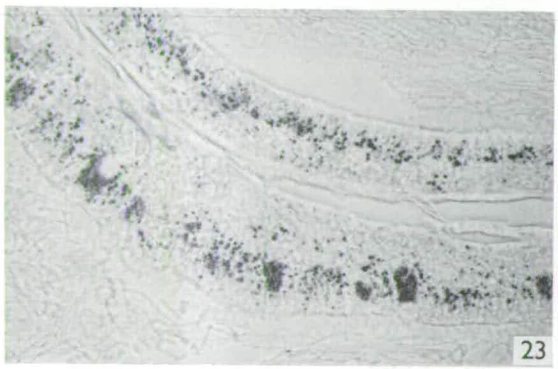
Figs. 18, 19. Rat epididymis. β-glucuronidase, hexagonium pararosanilin method, incubation time 30 minutes. × 250.

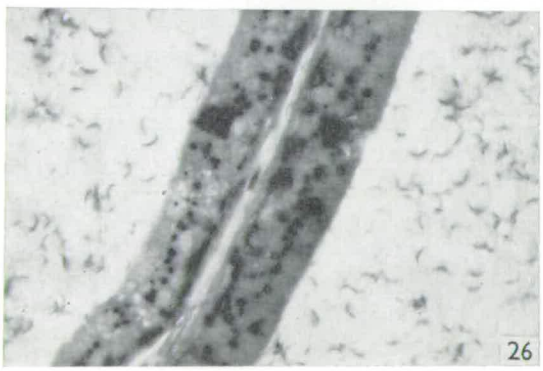
Fig. 18. Distal part of the middle segment.

Fig. 19. The terminal segment.

Fig. 20. Rat epididymis. Terminal segment. Non-specific esterase α-naphthyl method, incubation time 10 minutes. × 250.







Figs. 21–23. Hamster epididymis. Acid phosphatase, lead nitrate method, incubation time 10 minutes. × 750.

Fig. 21. Initial segment.

Fig. 22. Proximal part of the middle segment.

Fig. 23. Proximal part of the terminal segment.

Figs. 24, 25. Hamster epididymis. β-glucuronidase, hexagonium pararosanilin method, incubation time 30 minutes. × 250.

Fig. 24. Proximal part of the terminal segment.

Fig. 25. Distal part of the terminal segment.

Fig. 26. Hamster epididymis. Proximal part of the terminal segment. Non-specific esterase, α-naphthyl method, incubation time 10 minutes. × 750.

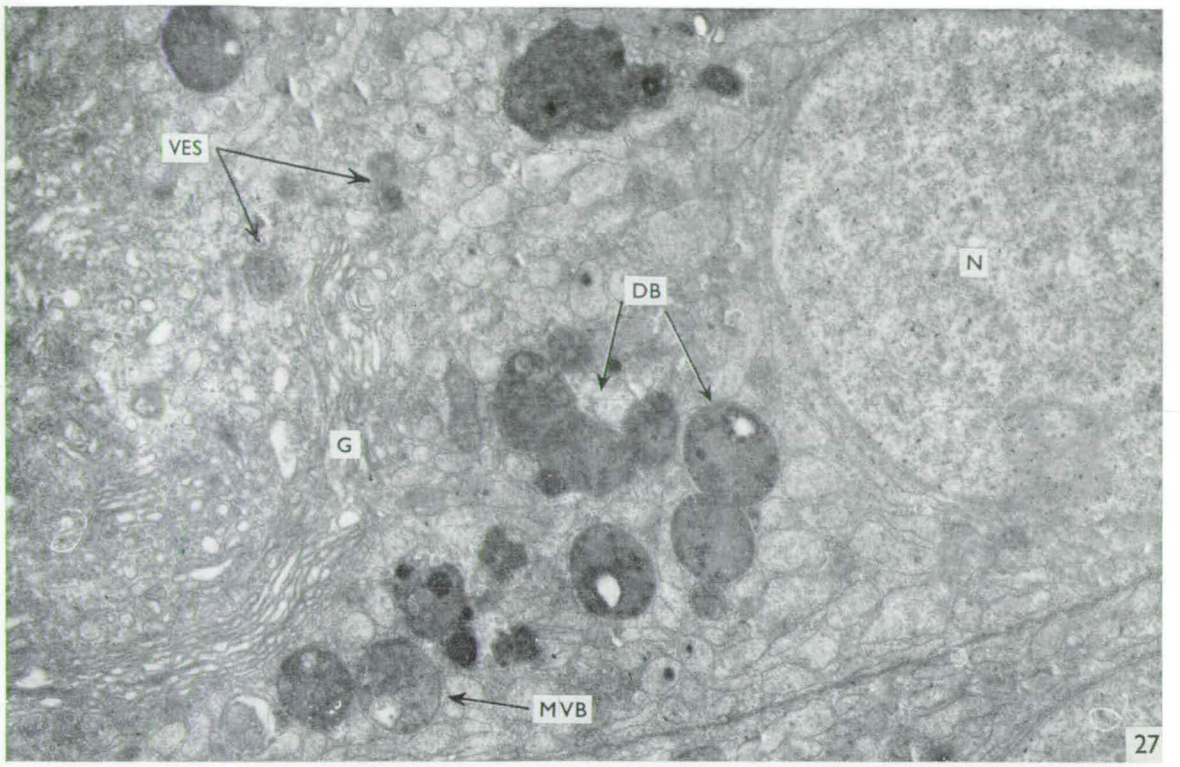
Although certain parts of the epididymis were rich in all three enzymes, the intensity of reaction or the number of granules stained for each similar enzyme was found to differ between species and between different regions of the epididymis in any one species. In general, the ductuli efferentes in all four species had low acid phosphatase activity, but rather high activity for β-glucuronidase and non-specific esterase. In the epididymis itself the proximal part of the middle segment in ram and rabbit, the distal part of the middle segment in rat, and the proximal part of the terminal segment in hamster all showed the highest activity for the lysosomal enzymes. Linnetz & Amann (1968) reported that the columnar cells of the proximal part of the middle segment (caput epididymidis) of the rabbit had the highest acid phosphatase activity, but the presence of columnar cells with extremely intense activity, also reported by these workers, was not confirmed in the present study. However, hamster and rat epididymides were characterized by the presence of such intensely staining cells, indicating very strong activity of lysosomal enzymes. The concentration of lysosomal enzymes in the middle segment of ram, rabbit and rat epididymis was in agreement with the finding of Glover & Nicander (1971) that spermatozoa were crowded in this region, and with their suggestion that many cytoplasmic vacuoles contained material absorbed from the luminal contents. In hamster, however, the lysosomes were concentrated in the proximal part of the terminal segment.

The presence in the connective tissue and in the luminal contents of some cells containing β-glucuronidase activity is of interest. It is possible that these cells are macrophages which migrate through the epithelial cells into the lumen and may have a role in the removal of remnants of degenerated spermatozoa or other debris, for Hayashi (1967) has reported that cells of the reticulo-endothelial system display high β-glucuronidase activity.

Both biochemical and cytochemical studies (de Duve, 1963; Novikoff, 1963; Gahan, 1967) have shown lysosomes to be a group of particles with a single limiting membrane and to be associated with a number of acid hydrolases. Acid phosphatase, β-glucuronidase and non-specific esterase are involved in the metabolism of phosphates, mucopolysaccharides and lipids respectively (Tappel, 1969). It is, therefore, possible that these enzymes are involved in the catabolism of substances that are absorbed from the epididymal lumen. The results of Burgos (1964, 1967), using colloidal mercuric sulphide, and of Sedar (1966) and Friend & Farquhar (1967), using peroxidase, are in agreement with the above suggestion. These authors have shown that the epithelial cells absorb particulate compounds from the duct lumen by pinocytosis and sequester the absorbed material in multivesicular bodies or lysosomes. Some authors (Lake, 1962; Martan, 1969) suggest that acid phosphatase found in the epithelial cells may be a secretory product that contributes to the enzyme found in the epididymal fluid. But the total absence of stained granules close to the

Fig. 27. Electron micrograph of supranuclear region of an epithelial cell from the initial segment of the epididymis of the hamster showing acid phosphatase activity in dense bodies (DB), multivesicular bodies (MVB) and Golgi associated vesicles (VES). N, nucleus, G, Golgi apparatus. Lead nitrate method. ×12000.

Fig. 28. Electron micrograph illustrating fine structural features of a similar area to that shown in Fig. 27. N, nucleus; G, Golgi apparatus; MVB, multivesicular body; DB, dense body. ×12000.


VES
N
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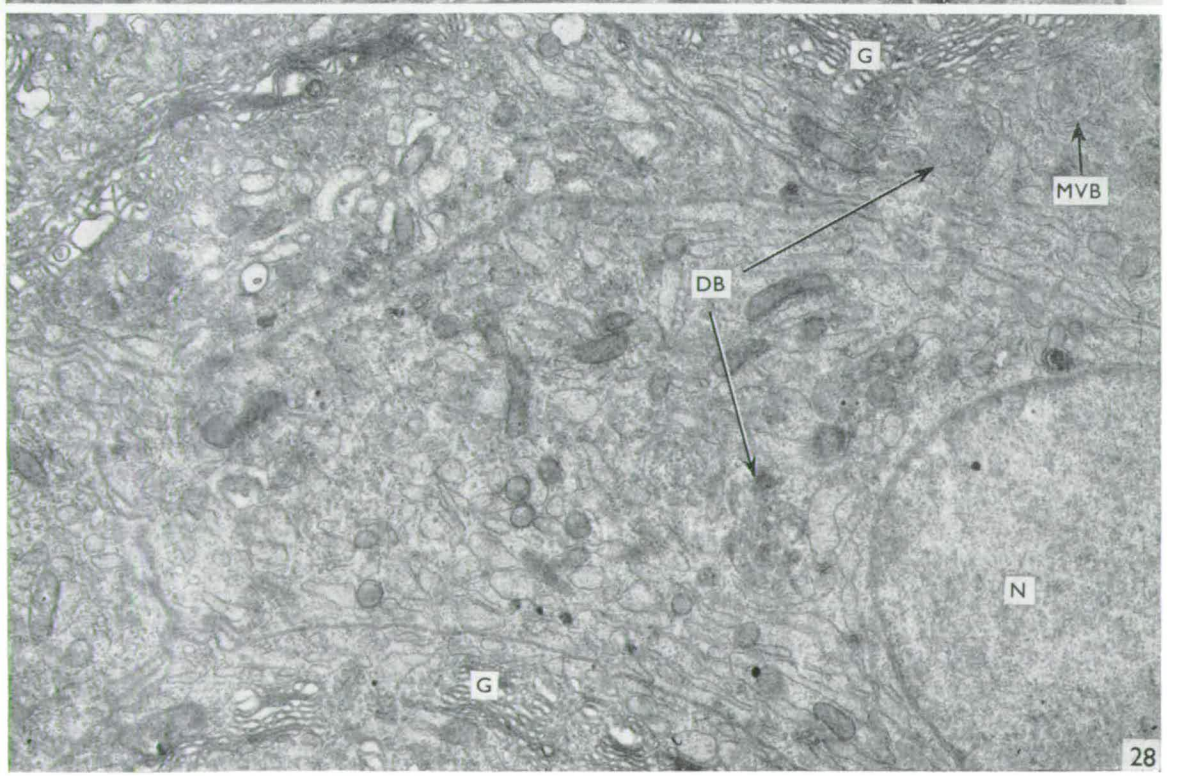
G
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28

luminal surface suggests, rather, that these enzymes are concerned with absorption. Fluid collected from the ductuli efferentes of slaughtered rams contains a low level of acid phosphatase, which increases to a high level in the terminal segment (Jones, unpublished observation). Whatever its origin, the acid phosphatase in testicular fluid may contribute significantly to the epididymal plasma, particularly in view of the concentration effect of the epididymis by selective absorption (Crabo, 1965). Furthermore, the studies carried out by Dott & Dingle (1968) on the lysosomal enzymes of the spermatozoa and cytoplasmic droplet of the bull and ram, and by Allison & Hartree (1970) on lysosomal enzymes of the acrosome, suggest that the spermatozoa and cytoplasmic droplet may contribute substantially to the phosphatase activity in the seminal plasma. These workers have shown, for instance, that alteration of osmolarity or pH of the suspending fluid causes disruption of the droplet, and it is realistic to predict that this may release the sequestered enzymes (Dott & Dingle, 1968).

SUMMARY

Using glutaraldehyde fixed tissues the histochemical localization of acid phosphatase, β-glucuronidase and non-specific esterase was studied in epididymides of the ram, rabbit, rat and hamster. The intracellular distribution of acid phosphatase in the epididymis of the hamster was also examined by electron microscopy. It was found that both acid phosphatase and β-glucuronidase were confined to discrete granules, while non-specific esterase was both cytoplasmic and granular in distribution. Granules stained for each enzyme were localized at similar sites, suggesting that they represent lysosomes. This was confirmed by electron microscopy. However, the intensity or number of these granules varied in the species studied, as well as in different regions of the ductus epididymidis. In general, the middle segment of the duct in the ram, rabbit and rat and the proximal part of the terminal segments in hamster epididymides showed the highest activity for all three lysosomal enzymes. The possible contribution of these enzymes to the function of the epididymis is briefly discussed.

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Regional histology and fine structure of the epididymal duct in the golden hamster (*Mesocricetus auratus*)

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INTRODUCTION

Conspicuous regional differences in the structure of the epididymal epithelium were described in dogs by Hammar (1897), and, much later, Benoit (1926) showed that the first part of the epididymal duct, which he called the 'initial segment', has characteristic features common to a series of mammals. In guinea-pigs, Laurent (1933) added a secretory zone and a reservoir zone, and Nicander (1957, 1958) found that, basically, Hammar's (1897) subdivision of the epididymal duct could be used for rabbits, stallions, bulls and rams, though in these species six to eight different regions were described. In rats, Reid & Cleland (1957) introduced a different histological subdivision into six zones, whilst Holstein (1969) described eight regions in the human epididymis. It is thus evident that the arbitrary terms caput, corpus and cauda epididymidis, used in gross anatomy to differentiate regions of the duct, are inadequate for comparative studies of epididymal function, since specific activity of the lining cells of the duct, such as secretion and absorption, often appears to be restricted to well-defined regions. Thus, if these conventional terms are used there may be marked differences in function within any given area. Glover & Nicander (1971) therefore suggested that the epididymis be divided into three main regions: the initial segment, the middle segment, and the terminal segment. These segments are based on histological, cytological and functional characteristics of the duct and do not correspond entirely to the caput, corpus, and cauda epididymidis in each species. It was previously mentioned by these workers that it might be possible to subdivide the hamster epididymis in this way and the present work is in part an attempt to assess the possibility. The epididymis of the hamster is in many ways more standard than that of the guinea-pig or mouse (Glover & Nicander, 1971), and thus the hamster is a particularly good rodent for use in epididymal research. It is therefore of some importance that the detailed structure of the hamster epididymis should be known, and since a comprehensive histological and cytological description of it appears to be lacking, the present work has been undertaken. The paper deals mainly with regional histology and the regional cytology of columnar cells with stereocilia.

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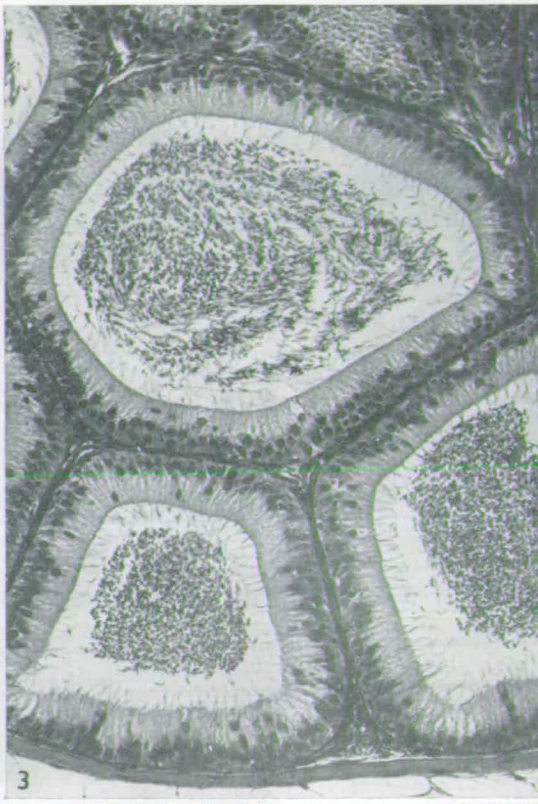
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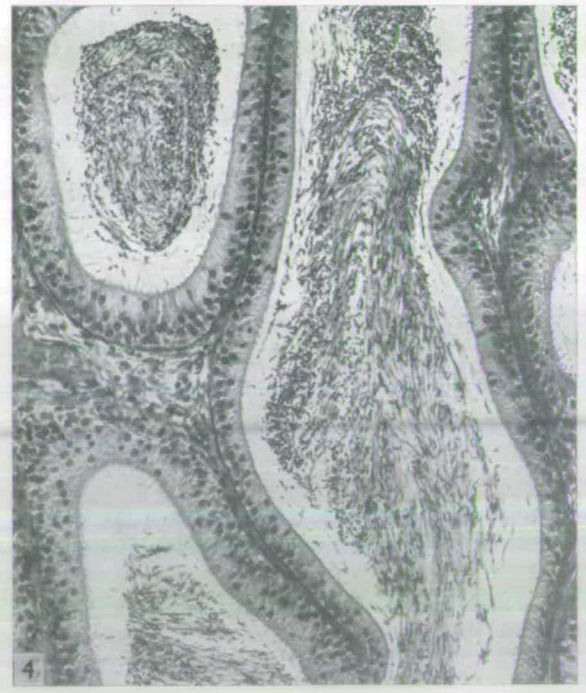
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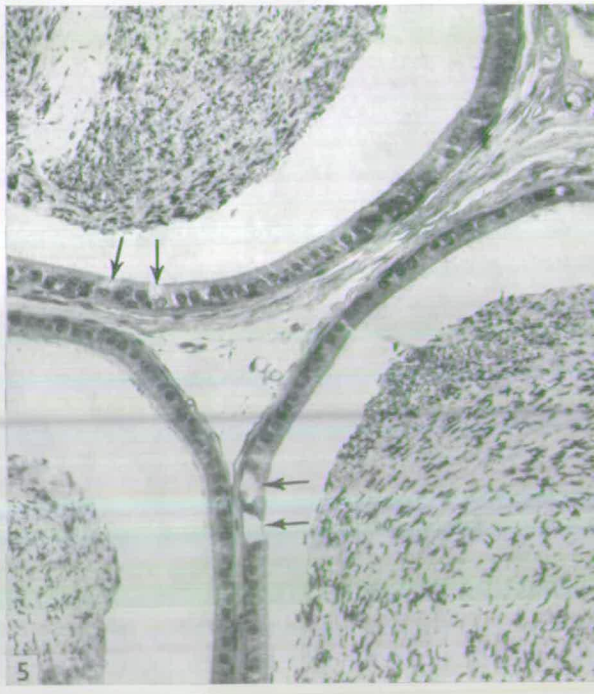
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MATERIALS AND METHODS

Twelve young, sexually mature golden hamsters were used. For histology one epididymis was fixed *in situ* in Bouin's fluid and embedded in paraffin wax. Longitudinal serial sectioning was performed, and selected sections were stained with haemalum and eosin. For cytology, small pieces were taken with a razor blade from different parts of the contralateral epididymis and fixed for 2 hours in a 1 % osmium tetroxide solution in Millonig's phosphate buffer with sucrose, or overnight in a 3 % phosphate buffered glutaraldehyde solution and then transferred to osmium tetroxide. Material from four animals was fixed by vascular perfusion with glutaraldehyde and pieces of epididymis were transferred to glutaraldehyde overnight and then treated as described above. All material was embedded in Epon. Semi-thin sections were cut for light microscopy and stained with toluidine blue; after selection of suitable areas, the blocks were trimmed and thin sections were cut with a diamond knife. These were stained with uranyl acetate, and sometimes with lead citrate also, before being examined with a Siemens Elmiskop I or a JEM 7A electron microscope at magnifications between 1500 and 11000 diameters.

OBSERVATIONS

Light microscopy

The initial segment (*Figs. 1, 7*)

This shows the usual characteristics, namely a large overall diameter of the duct and a high epithelium with very long stereocilia that almost fill the lumen. Some spermatozoa are present in the lumen, and most of them are to be seen in the posterior part of the segment. The oval nuclei of the columnar cells are generally situated just below the centre of the cell, and the infranuclear cytoplasm shows a conspicuous basophilia. Above the nucleus, there is a very large Golgi area, separated from the pale apical cytoplasm by a narrow band of basophilic cytoplasm. In Epon sections, many mitochondria are to be seen in this band. Numerous small dense granules are seen between the Golgi apparatus and the nucleus, and some occur along the periphery of the Golgi area. In the posterior part of the initial segment a few vacuoles appear in the apical cytoplasm. Scattered light columnar cells with their nucleus in the apical half of the cell are also seen, and, in addition to the true basal cells, small light cells are seen either basally or between the columnar cells at higher levels of the epithelium.

Figs. 1–5. Photomicrographs of paraffin sections of a hamster epididymis. Bouin fixation, H. & E. × 160.

Fig. 1. Initial segment.

Fig. 2. Middle segment, intermediate part.

Fig. 3. Middle segment, distal part (proximal corpus).

Fig. 4. Terminal segment, proximal part.

Fig. 5. Terminal segment, distal part (middle cauda). Four light cells are indicated by arrows.

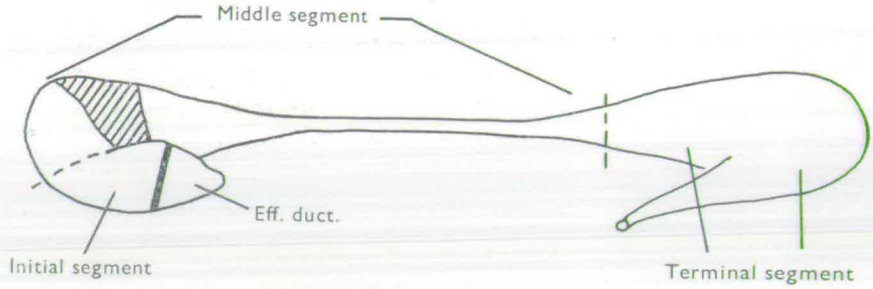

Fig. 6. Regions of the epididymis in the hamster. Cross-hatching indicates intermediate part of middle segment.

The middle segment (*Figs. 2, 3, 8, 9*)

This is characterized by the presence of distinct vacuoles in the apical cytoplasm. The diameter of the duct is smaller and the epithelium is lower than in the initial segment. The stereocilia are also considerably shorter. The nuclei of the columnar cells are oval and situated basally, and they are surrounded by basophilic cytoplasm. In the Epon sections, the Golgi area is seen to be smaller than in the initial segment, and somewhat indistinct. A band of dense cytoplasm with mitochondria separates the Golgi area from the apical band of clear cytoplasm and the vacuoles. Dense bodies are mostly to be seen in the narrow area between the Golgi apparatus and the nucleus. As in the initial segment, basal cells and small light cells are present.

Three subdivisions of the middle segment are recognized (Fig. 6). The proximal part shows most epithelial vacuoles and a moderate number of spermatozoa in the lumen. The intermediate part shows a narrower duct, a lower epithelium, and fewer epithelial vacuoles, and in this area the crowding of spermatozoa in the lumen is striking. The posterior part (the posterior caput and corpus epididymidis) is similar to the proximal part in terms of duct diameter, epithelial height, and moderate numbers of spermatozoa, but vacuoles are few or rare. In the posterior corpus epididymidis, the apical band of light cytoplasm is often invaded by mitochondria, and the border between the middle and terminal segments is not as distinct as that between the initial and middle segments; in some hamsters it seemed, in fact, to be within the corpus rather than at the corpus-cauda junction.

Figs. 7–11. Photomicrographs of Epon sections from a hamster epididymis. Fixed by vascular perfusion with glutaraldehyde, followed by osmium tetroxide. Toluidine blue. See text. × 1200.

Fig. 7. Initial segment, with long coarse stereocilia, large Golgi area (*GA*), columnar cell nuclei at a high level, and numerous supranuclear granules. Three small light cells are indicated by arrows.

Fig. 8. Middle segment, intermediate part. Note densely crowded sperm in lumen, low stereocilia, and the mainly apical vacuoles. The Golgi apparatus (*GA*) is of moderate size.

Fig. 9. Middle segment, distal part, with few vacuoles but numerous mitochondria in apical cytoplasm. Moderate numbers of spermatozoa in the lumen.

Fig. 10. Terminal segment, most anterior part. Lower columnar cells with apparently homogeneous cytoplasm.

Fig. 11. Terminal segment, distal part (middle cauda). Low epithelium with dense cytoplasm and conspicuous Golgi area. One light, vacuolated columnar cell is also seen.

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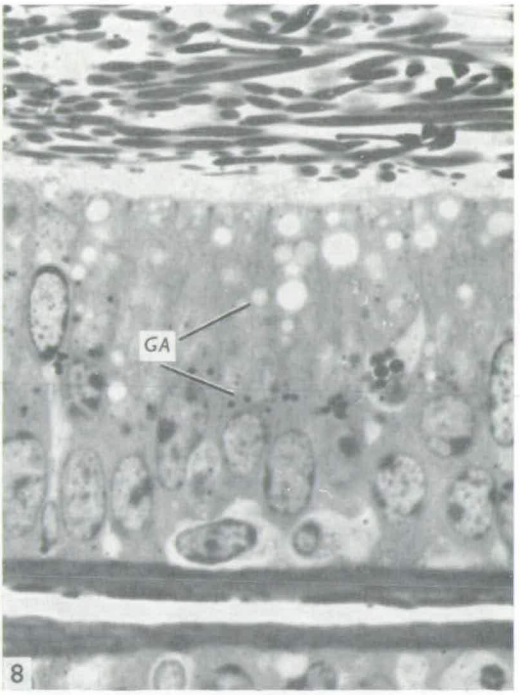
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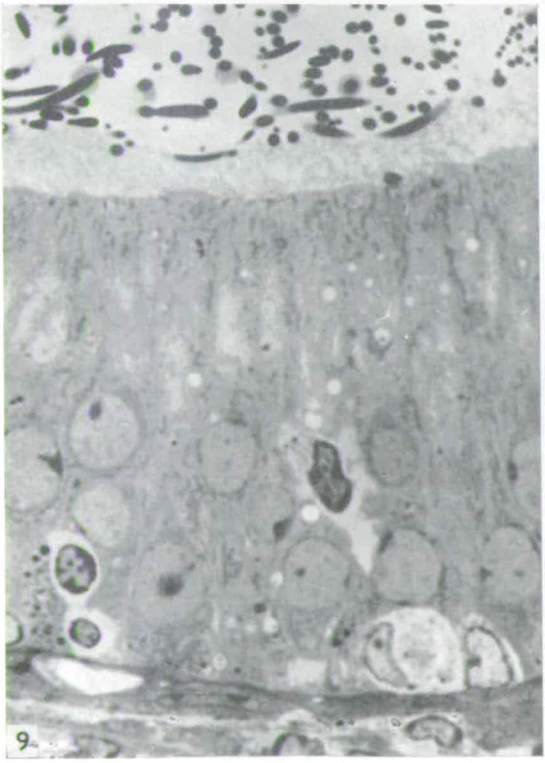
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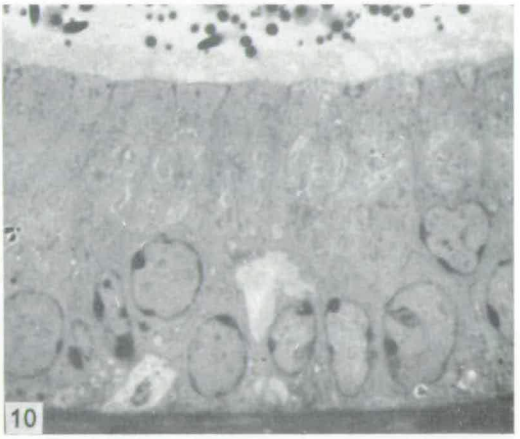
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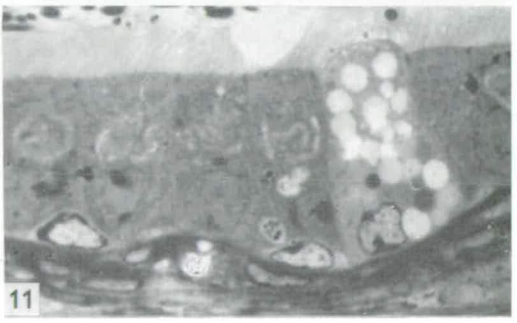
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The terminal segment (*Figs. 4, 5, 10, 11*)

This segment may be subdivided into two parts. The proximal one still shows a high epithelium, and generally the columnar cell nuclei occur at varying levels. The distal part (most of the cauda) shows a lower epithelium, a large overall diameter of the duct, and a wide lumen. The stereocilia are low. The rounded nuclei are situated basally and cytoplasmic basophilia is less conspicuous than in other segments. The Golgi area is very distinct in the distal part of the segment, and it tends to be apical in position. The apical cytoplasm is dark and homogeneous, but small vacuoles and granules are often seen in the Golgi area. Granules of varying size are more numerous in the cytoplasm between the Golgi area and the nucleus, especially in the proximal part of the segment. Mitochondria are common except in the Golgi area. Basal cells and numerous small light cells are also seen and light vacuolated columnar cells without stereocilia are also frequent in this segment.

Electron microscopy

Most ultrastructural features are common to the stereociliated columnar cells of all segments. The stereocilia are microvilli which have a core of fine filaments running longitudinally through the villus and often continuing for some distance into the apical cytoplasm. The lateral cell borders are often winding or folded and prominent terminal bars are always present. Numerous microtubules often radiate into the cytoplasm from the terminal bar, and sometimes they even penetrate into the centre of the Golgi area. Bundles of dense filaments are also seen in most of the columnar cells after glutaraldehyde fixation. The endoplasmic reticulum is very abundant in all regions and is generally somewhat granular, except where it occurs in an apical band of cytoplasm. It is characterized by its opaque contents. Free ribosomes are common, and the mitochondria are elongated and have many transverse cristae and a dense matrix. The Golgi complex is always large and built of several stacks of cisternae, most of them in the periphery but some in the interior of the area. Numerous small Golgi vesicles are also seen, as well as a few small coated vesicles, sometimes in continuity with a Golgi cisterna. The cytoplasm in the centre of the Golgi area is poor in ribosomes and mitochondria, but it contains many small vesicles and often also some clear vesicles of moderate size. Generally, however, these clear vesicles are only distinct after glutaraldehyde fixation. Vacuoles, multivesicular bodies, and dense bodies of various types may also be present in the Golgi area. Pictures suggesting micropinocytosis are also to be found. For instance, there are invaginations of the apical plasma membrane with a characteristic thickening of the membrane, and juxtaluminar vesicles with a similar thick membrane (Figs. 13, 16). The apical cytoplasm also contains vesicles of moderate size, best seen after glutaraldehyde fixation, and these are probably of pinocytotic origin, although some of them are clear, like the vesicles in the Golgi area and might be secretory. After good fixation, there is never any evidence of apocrine secretion.

Regional characteristics

At the ultrastructural level these are less easily defined than under the light microscope. The *initial segment* (Figs. 12, 13, 14) shows rather thick, irregular stereo-

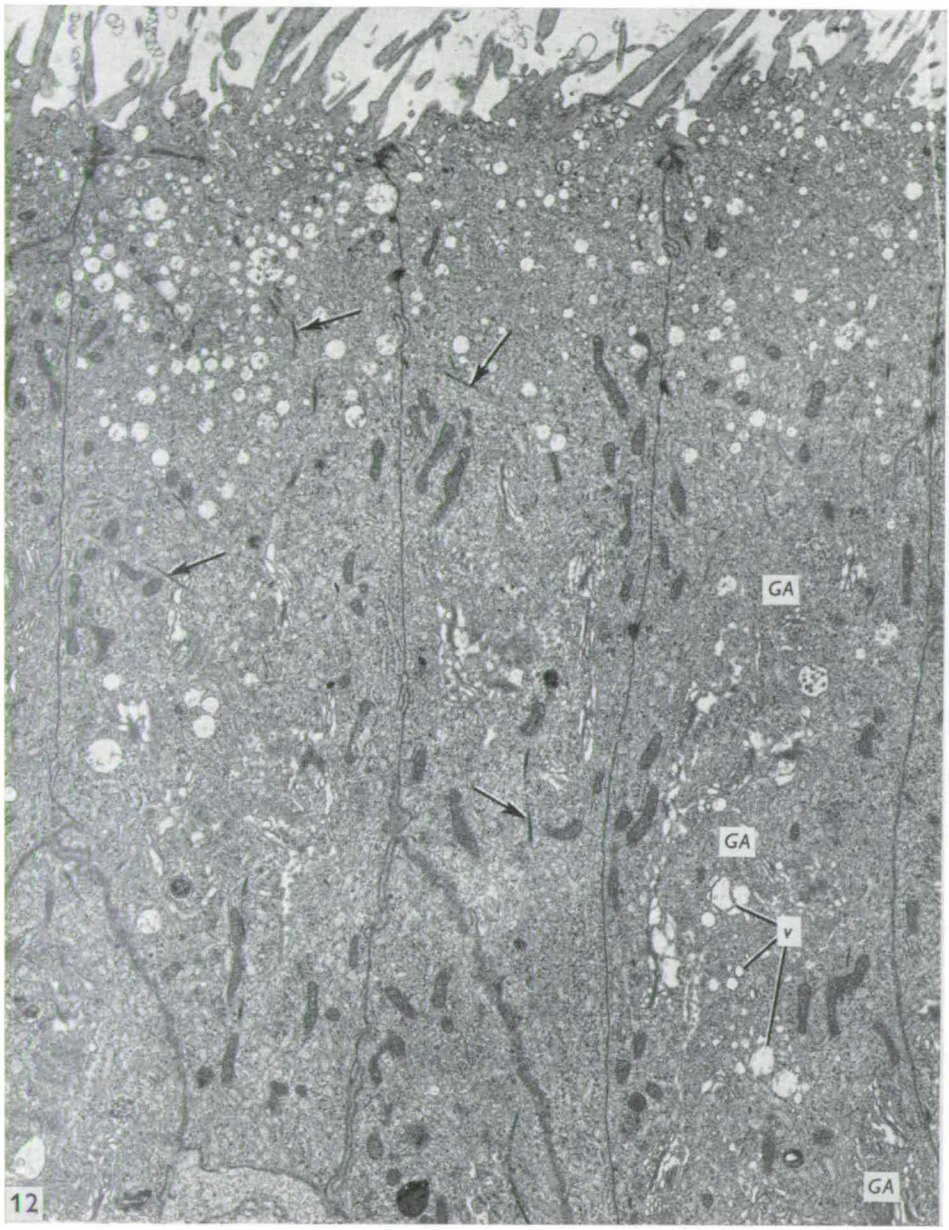
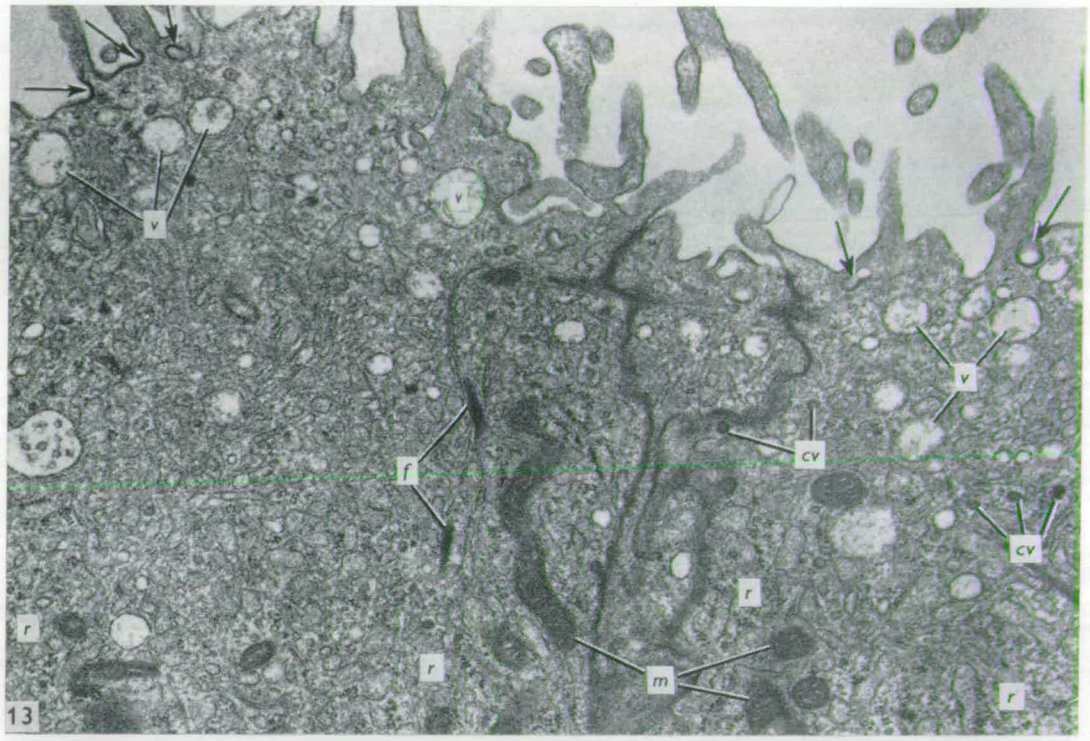


Fig. 12. Electron micrograph showing apical parts of columnar cells in initial segment. Note coarse stereocilia and numerous vesicles in apical cytoplasm. The large Golgi complex (*GA*) with narrow cisternae contains some clear vesicles (*v*). Some bundles of filaments are also seen (arrows). Perfused with glutaraldehyde. × 6000.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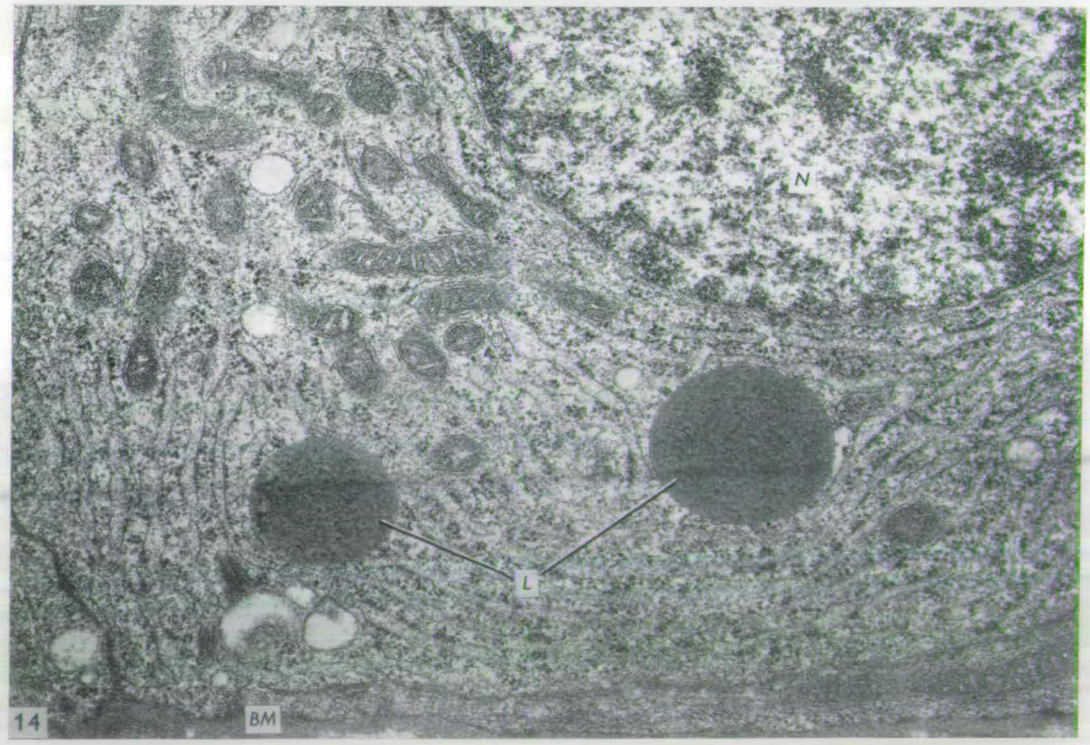
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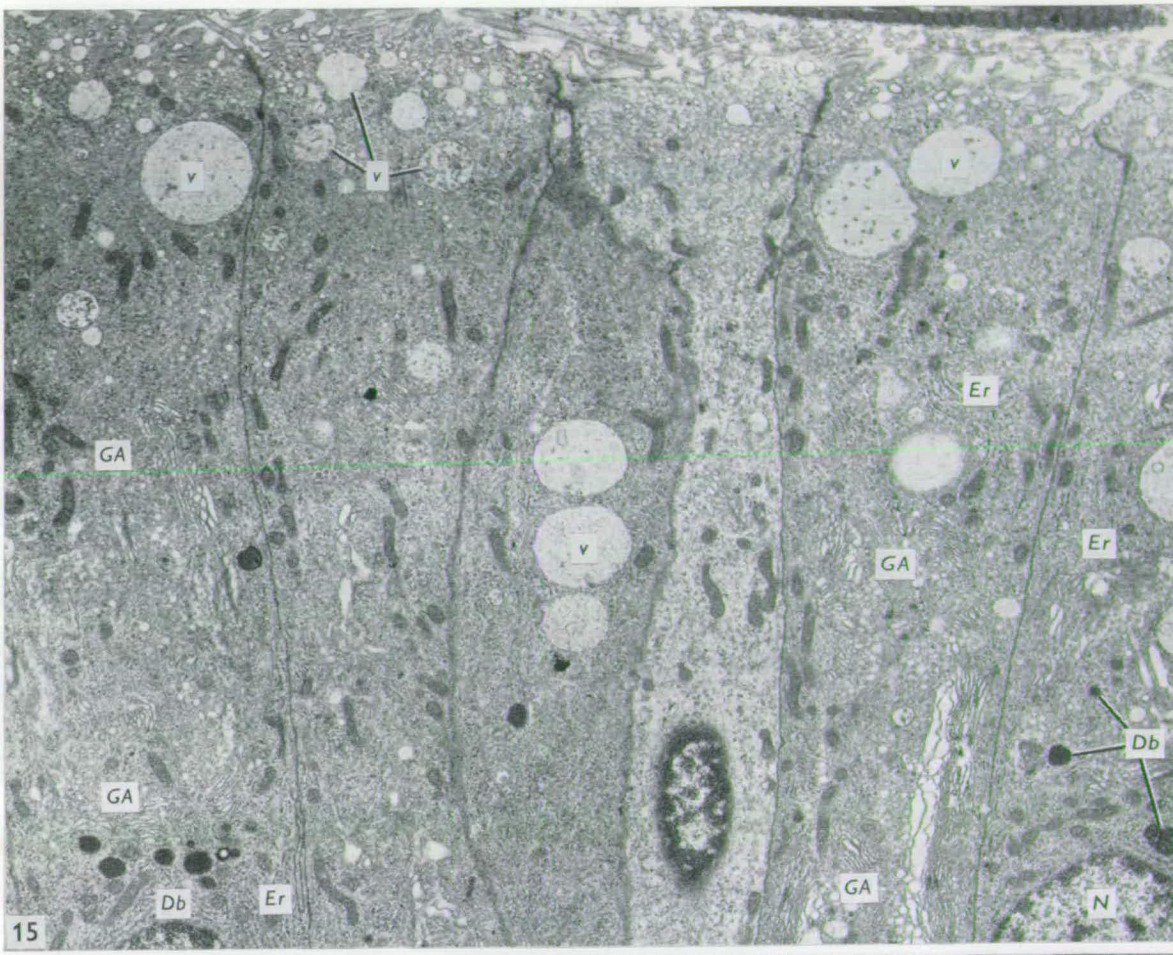
cilia, whilst the apical rim of cytoplasm is generally free of mitochondria and ribosomes, and is crammed with rounded and elongated profiles of smooth endoplasmic reticulum. Some micropinocytotic invaginations of the plasma membrane are always seen, but the vesicles thus formed apparently lose their membrane thickening after being pinched off from the plasma membrane. Many larger vesicles, spherical after glutaraldehyde fixation, are seen in the apical cytoplasm, and they often contain aggregates of an electron-dense substance. In some cases, such material is also present in the adjacent lumen and in the pinocytotic invaginations of the apical plasma membrane. Apparently they are taken up from the lumen by pinocytosis. A few vesicles of this type are also seen just above and within the Golgi area, but here multivesicular bodies are more common. Dense bodies of moderate size are rather numerous just below the Golgi complex. The endoplasmic reticulum is largely rough-surfaced, particularly in the cytoplasm immediately above and below the Golgi area, and it seems to be exclusively rough around the nucleus and in the broad zone of infranuclear cytoplasm. Whilst free ribosomes are rare, small lipid droplets are often to be seen in the basal cytoplasm.

The *middle segment* (Figs. 15, 16) also shows thick, irregular, sometimes anastomosing microvilli. The apical cytoplasm is characterized by few ribosomes, the virtual absence of mitochondria, and an abundant smooth endoplasmic reticulum which is apparently more vesicular than that in the initial segment. Signs of pinocytosis are frequent and many vesicles with the characteristic membrane thickening occur in the juxtaluminar cytoplasm. The most conspicuous structures are the vacuoles, apparently formed by the confluence of pinocytotic vesicles, since their boundary membrane often shows similar thickened areas (Fig. 16). Their contents are opaque and flocculent, and many vacuoles also contain varying numbers of small internal vesicles or bodies. Vacuoles may also be seen below the apical area, but in the Golgi area only a few multivesicular bodies and dense bodies are present. More dense bodies occur in the narrow band of cytoplasm between the Golgi apparatus and the nucleus. In both the supranuclear and infranuclear areas some cisternae of the endoplasmic reticulum show only a few attached ribosomes, and free ribosomes are much more abundant than in the initial segment, especially just above the Golgi area. Small coated vesicles are more numerous than in the initial segment, both in the Golgi area and in the apical cytoplasm. Small lipid droplets may also be present below the nucleus.

In the posterior part of the middle segment (corpus epididymidis) vacuoles are less numerous, and may be very rare; they occur at deeper levels of the cell, and may even be below the nucleus. The apical band of light cytoplasm is very narrow and often contains vesicles similar to the apical vesicles of the initial segment. Pinocytosis still appears to occur rather extensively, and multivesicular bodies and

Fig. 13. Part of two cell tops, initial segment. The ground cytoplasm is crowded with smooth membranes. A few micropinocytotic phenomena (arrows), large vesicles (*v*) with some flocculent contents, mitochondria (*m*), free ribosomes (*r*), small coated vesicles (*cv*), and filament bundles (*f*). Perfusion with glutaraldehyde. × 20000.

Fig. 14. Basal cytoplasm of columnar cell in the initial segment, with prominent rough endoplasmic reticulum, mitochondria, and lipid droplets (*L*) below the nucleus (*N*). *BM*: basement membrane. Perfusion with glutaraldehyde. × 32000.



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moderately dense bodies are more numerous in and below the Golgi area than in higher levels of the middle segment.

The *terminal segment* (Figs. 17, 18) shows slender, regular microvilli. The ground cytoplasm appears to be denser than in the other segments, and good fixation is rare. Therefore, the endoplasmic reticulum does not stand out clearly, but is very probably less well developed than in the other segments, and more areas are free of attached ribosomes. Free ribosomes, however, are common, and occur even in the juxtaluminar cytoplasm. Mitochondria are very numerous and invade the apical cytoplasm, whilst the bundles of dense filaments are few and thin. The Golgi complex is better demarcated from the surrounding cytoplasm than in other regions, and there are many very long cisternae in each stack, especially in the middle and posterior parts of the segment. Pinocytotic invaginations are few and small, and only few small vesicles with a thickened membrane are seen in the apical cytoplasm. Small coated vesicles also appear to be less frequent than in the middle segment, at least above the Golgi area. Clear vesicles are rare, even in the Golgi area, but multivesicular bodies and other spherical bodies with contents of varying density are conspicuous in the centre of the Golgi area. The broad zone between the Golgi apparatus and the nucleus contains many dense and composite bodies, and some of these are to be seen below the nucleus also. No lipid droplets are present.

DISCUSSION

Basically, the fine structure of the columnar epithelial cells in the epididymis of the golden hamster appears to be similar to that in the epididymis of the rabbit (Nicander, 1965), rat (Friend, 1969; Hamilton, 1971), and man (Holstein, 1969). The stereocilia are microvilli with a filamentous core, and the cytoplasm contains an extensive endoplasmic reticulum and a large Golgi complex. Except for the most apical cytoplasm in the initial and middle segments, all cytoplasmic areas show at least some ribosomes attached to the membranes of the reticulum. As in rats (Hamilton, 1971) and rabbits (Nicander, 1965), the epididymal cells of the hamster seem to possess machinery for protein synthesis that is far in excess of what is necessary for the production of lysosomal enzymes or for enzyme secretion. The smooth endoplasmic reticulum is of special interest considering the earlier view of Hamilton & Fawcett (1970) and Hamilton (1971) that the smooth endoplasmic reticulum of the epididymal duct epithelium of mice and rabbits might actively participate in the biosynthesis of steroids. The position here is a little confused, however, because it seems that this concept has been recently modified (Hamilton, 1972).

Fig. 15. Supranuclear parts of columnar cell in the intermediate part of the middle segment, showing vacuoles (*v*) above, and dense bodies (*Db*) below the Golgi complex (*GA*). *N*, nucleus, *Er*, rough endoplasmic reticulum. Perfusion with glutaraldehyde. × 7000.

Fig. 16. Juxtaluminar cytoplasm of columnar cell in the same region with numerous pinocytotic vesicles (arrows), vacuoles of various sizes, larger vesicles (*v*), and small coated vesicles (*cv*) in ground cytoplasm which is filled with membranes. *m*, mitochondria. Glutaraldehyde perfusion. × 20000.

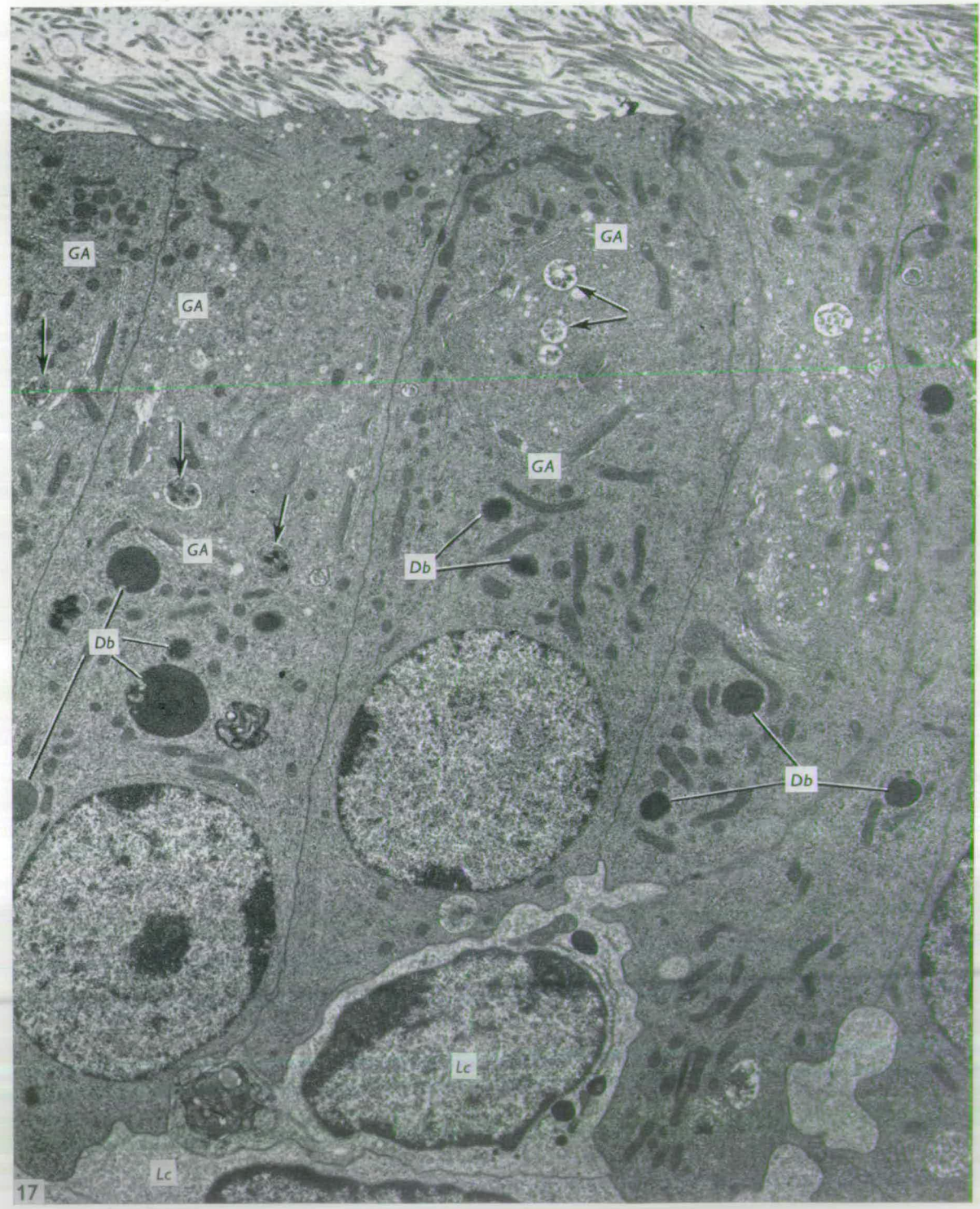

Fig. 17. Survey of columnar cells in terminal segment, proximal part. Note slender microvilli (stereocilia), mitochondria in cell apex, absence of vacuoles, compact Golgi area (*GA*) above the nucleus. Vacuoles (arrows) with dense contents also present, and dense bodies (*Db*). Small light cells (*Lc*) also. Glutaraldehyde perfusion. × 6000.

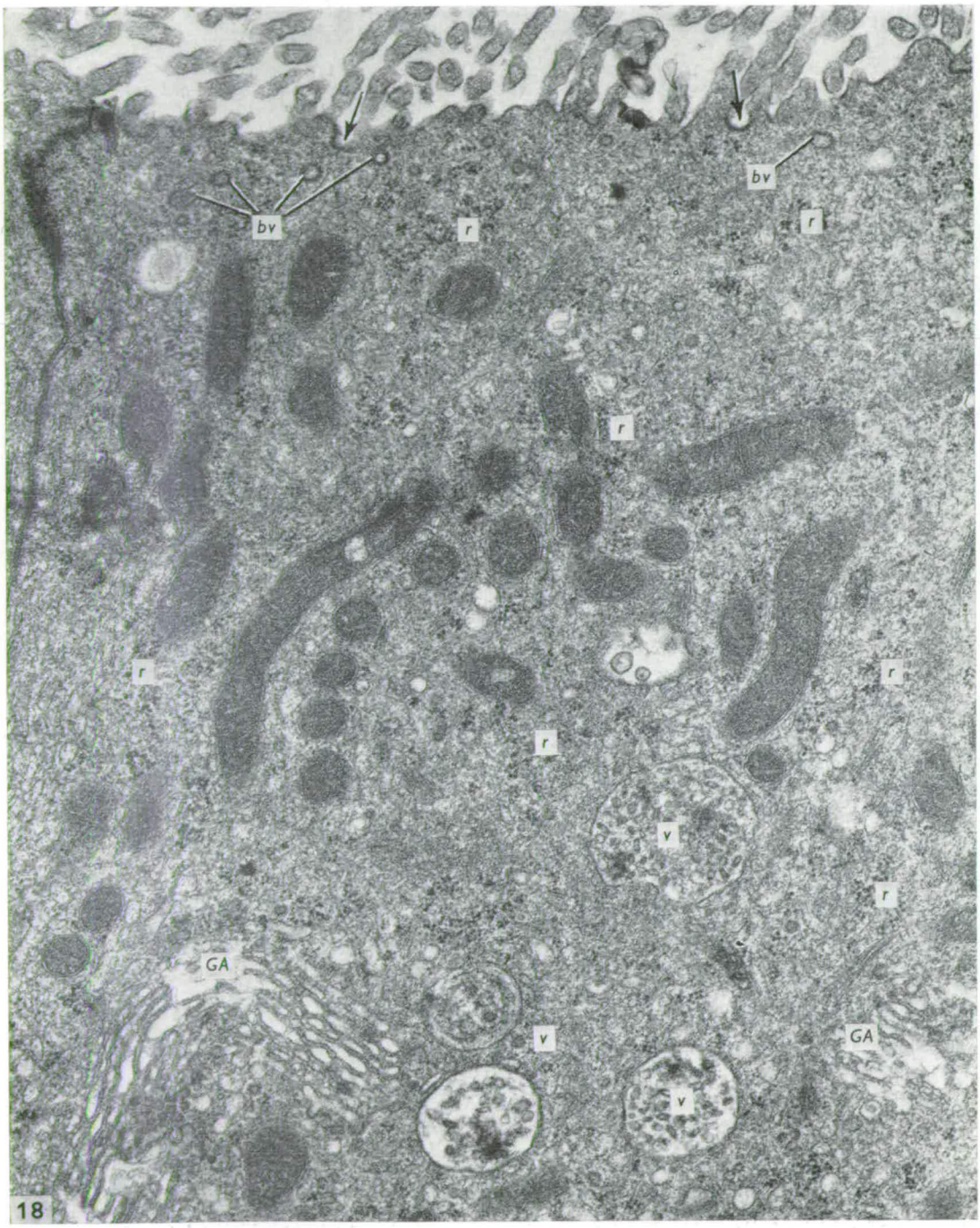

Fig. 18. Apical part of a columnar cell in terminal segment, distal part. A few micropinocytotic vesicles forming invaginations of apical plasma membrane (arrows) and bristle-coated vesicles (*bv*) in the compact ground cytoplasm which has prominent mitochondria. Near or in the Golgi area (*GA*) there are vacuoles (*v*) with internal 'vesicles' and dense material. *r*, ribosomes also shown. Perfusion with glutaraldehyde. ×26000.

A large Golgi apparatus is also a well-known feature of mammalian epididymal cells. It has been considered to take part in a secretory process of serous character, but this concept has not received any support from recent research (cf. Nicander, 1970). On the other hand, the mammalian epididymis contains numerous lysosomes (Nicander, 1965, 1970; Novikoff, Hirsch & Quintana, 1965; Friend, 1969; Köhl, 1968; Moniem & Glover, 1972), and the Golgi apparatus is considered to take part in their formation. The transport of acid phosphatase from the Golgi apparatus to one type of young lysosome (multivesicular bodies) by Golgi vesicles has been demonstrated by electron microscopical cytochemistry in the epididymis of rats (Friend, 1969) and rabbits (Nicander, unpublished observations) and although perhaps it might be more difficult to demonstrate, it is likely to occur in other species. Another probable activity in epididymal cells is the participation of the Golgi apparatus in the secretion of enzymes (see below) and other substances. After glutaraldehyde fixation, the interior of the Golgi area in rabbit and ram epididymides contains numerous rather large, clear vesicles, and similar vesicles are also seen in the apical cytoplasm (Nicander, unpublished observations). The clear vesicles observed in similar locations in the initial and middle segments of the hamster epididymis are obviously of the same type. It is just possible that the vesicles described here may represent a mode of secretion, and if this were true, more dramatic signs of secretion are not likely to be observed. At present, of course, this must be speculative. The old concept of an abundant apocrine secretion in the epididymis was not supported by Hammar (1897) or Benoit (1926), and modern methods of fixation have, so far, demonstrated that any such signs are artifacts (Nicander, 1970). Moreover, the 'secretory' vacuoles and granules of classical cytology have been shown to be pinocytotic vacuoles and lysosomes respectively (cf. Nicander, 1970). This also holds true for the hamster epididymis (Burgos, 1964; Sedar, 1966; Moniem & Glover, 1972). However, the secretion of alkaline phosphatase has been demonstrated histochemically in the epididymal tail of hamsters (Malone & Bower, 1962; Moniem, 1972) and biochemically in the epididymal tail of boars (Einarsson, 1971), and both alkaline phosphatase and lysosomal enzymes are secreted in the terminal segment of rabbits (Jones & Glover, 1973). The clear vesicles mentioned above may take part in the secretion of these and other substances as suggested by Nicander (1970), though few of these vesicles are seen in the proximal part of the terminal segment in the hamster where alkaline phosphatase is secreted.

The concept that the vacuolated columnar cells are holocrine has recently been refuted (Clermont & Flannery, 1970) and will not be discussed here.

Evidence of micropinocytosis was certainly seen in all regions of the epididymis, although it appeared to be more extensive in the middle segment than in the initial segment, and occurred only infrequently in the terminal segment. The uptake of tracers from the lumen in the head of the epididymis has already been demonstrated in hamsters (Mason & Shaver, 1952) and it has also been shown to take place by means of micropinocytosis both in the initial segment (Sedar, 1966) and in the middle segment (Burgos, 1964). Similar observations have been made in the terminal segment of rabbits (Nicander, Paulsson & Selander, 1966) and rats (Friend & Farquhar, 1967; Friend, 1969). The tracers used were either a peroxidase or iron-carbohydrate compounds and, as has been seen in other species (Nicander, 1966), they were

transported into and digested in lysosomes, often having multivesicular bodies as intermediate stages.

In the middle segment of the rabbit epididymis, opaque material occurring normally in the lumen is taken up by pinocytosis and transported into lysosomes (Nicander, 1965). Friend & Farquhar (1967) and Moniem & Glover (1972) have suggested that a similar pinocytosis-lysosomal mechanism occurs in the epididymis of the rat and the hamster. In the present study, some uptake of material from the lumen appeared to take place in the initial segment. But although pinocytotic phenomena, multivesicular bodies and lysosomes are well recognized in some mammalian epididymides (Nicander, 1970; cf. Moniem & Glover, 1972), their precise functional significance is not clearly understood and further experimental work is needed to close this gap in our knowledge.

Unlike higher regions of the duct, where spermatozoa are undergoing maturation, the terminal segment of the epididymis is concerned with the storage of spermatozoa, and there is some evidence that the lining cells in this area may not be as metabolically active as those higher up the duct (Turner & Johnson, 1973; White, Wallace, Wales & Scott, 1961). Nevertheless, interpretations should be made with caution, because activity of the cells in this area might be influenced by a number of factors, which would not impinge on cells at a higher level. The frequency of ejaculation, for instance, does not apparently influence activity above the terminal segment, but Vitale-Calpe & Aoki (1970) claimed that in the hamster the cells of the terminal segment become taller 1 hour after coitus. Apparently the Golgi complex also becomes smaller and dense granules disappear. The level of the terminal segment requires definition also, because in hamsters it has been found that phosphorylase activity is highest in the proximal part of the cauda epididymidis. Fouquet & Guha (1969) and Moniem & Glover (1972) have demonstrated that three lysosomal enzymes show the highest activity in this same part of the terminal segment, a region where alkaline phosphatase is also actively secreted (Malone & Bower, 1962; Moniem, 1972). Radioactive phosphatase is also more rapidly incorporated into this part of the hamster epididymis than into any other region (Soderwall & Chaimov, 1954) and, in addition, there are many mitochondria in this part of the duct. Since spermatozoa become functionally mature in this region (Horan & Bedford, 1972) it certainly looks as if the cells of at least part of the terminal segment are very active indeed.

The suggestion of Glover & Nicander (1971) that the epididymis of the hamster might, together with that of several other mammals, be conveniently sub-divided into initial, middle and terminal segments, is generally confirmed by the present more detailed work on this species. But the border between the middle and terminal segments in the hamster is now more clearly defined, and from a functional standpoint this is quite important, because it concerns the level of the duct at which spermatozoa finally reach maturity. In rabbits, for instance, it has been shown that spermatozoa do not reach full maturity until they have passed well into the terminal segment (Bedford, 1966; Orgebin-Crist, 1969).

Histochemical reactions in the epididymis may be confusing if they are used in an attempt to assess regional differences in function, because the picture presented differs even in the same species, according to the substances under investigation.

This is apparent in regard to the hamster, when the papers of McGadey, Baillie & Ferguson (1966) and Fouquet & Guha (1969) are compared. Also Fouquet (1970) reported the presence of glycogen in the infranuclear cisternae of the rough endoplasmic reticulum in the hamster epididymis, but we were unable to demonstrate it in our material.

Nevertheless, histochemical features can be extremely useful indications of function in a strictly defined area of the epididymis, and they have been used successfully in this manner (Nicander, 1957, 1958; Erkmann, 1971; Moniem & Glover, 1972).

The importance of regional differentiation is stressed here and the suggested terminology is recommended, because it can be correlated with maturation changes in the spermatozoa, that is, as far as the rabbit and the hamster are concerned. However, more species will yet have to be studied in this context before the validity of the sub-division can be fully evaluated.

SUMMARY

The regional histology and cytology of the epididymal duct in the golden hamster have been studied with light and electron microscopy. Three main regions are described: the initial segment, middle segment (from the caput flexure to the corpus-cauda junction), and terminal segment. The initial segment has a wide lumen, a high epithelium with long stereocilia, much ergastoplasm, and many apical vesicles. The middle segment has a lower epithelium with apical vacuoles. Three subdivisions are described: the proximal part has more vacuoles, the intermediate part a narrow lumen with densely crowded spermatozoa, and the distal part (corpus epididymidis) few vacuoles in a higher epithelium and fewer spermatozoa in the lumen. The terminal segment shows a wider lumen and a low epithelium with a dense cytoplasm without vesicles or vacuoles, and showing little pinocytosis. In the light microscope its proximal part appears to be rather similar to the middle segment but generally it stands out by virtue of an irregular arrangement of the nuclei.

The general fine structure of the columnar epididymal cell with stereocilia is described, and the possible importance of its characteristic features in pinocytosis, intracellular digestion, and secretion is briefly discussed.

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ASPECTS OF SPERM PRODUCTION IN SOME EAST AFRICAN MAMMALS

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Summary. A comparison of certain features of the testis and epididymis has been made in five species of East African mammals, two of which, the rock hyrax and elephant, have abdominally situated testes.

The artery to the testes was straight in the species with abdominally situated testes and there was no pampiniform plexus while in the species with scrotal testes, it was coiled in the region of the plexus. It is suggested that where the testicular artery is coiled, the testes should be regarded as basically scrotal, even if they are usually found in the abdomen *post mortem*.

A striking increase in blood flow in the testis of the rock hyrax during sexual activity suggests that the simpler arterial pattern of the testis in testicond mammals allows a greater variation in blood flow than the more complicated arterial design associated with scrotal testes.

Characteristic signs of sperm maturation occur in the epididymis of testicond mammals in contrast to the situation in artificial cryptorchidism, where normal epididymal function is completely disrupted. It is suggested that epididymal function, as well as spermatogenesis, has become modified during evolution. Evidence is given that a need for prolonged survival of spermatozoa in the mesonephric duct might have been an important primary factor in the caudal migration of gonads into a scrotum.

INTRODUCTION

In this paper, reference is made mainly to the testis and epididymal contents of the giraffe, *Giraffa*, eland, *Taurotragus*, steinbok, *Raphicerus*, jumping hare, *Pedetes*, rock hyrax or dassie, *Procavia* and *Heterohyrax*, and elephant, *Loxodonta*, because examples of all these genera are found in equatorial Africa and thus have several features of environment in common. In the first four genera, the testes are situated in a scrotum. In the hyrax and the elephant, they are permanently and normally in the abdomen (mammalian testiconda). The jumping hare has a scrotal pouch but the testes have been described as being abdominal (Coe, 1969).

In mammals in which the testes and epididymides are in a scrotum, the production of spermatozoa comprises spermacytogenesis, involving cell

division, and spermateliosis, involving no cell division. The process of sperm maturation, therefore, begins in the testis but is continued in the epididymis long after the spermatozoa leave the testis. It is well known that in scrotal mammals, spermatogenic activity of the testis is highly susceptible to elevated temperature. It has also been shown that maturation and survival of spermatozoa in the epididymis is extremely sensitive to increased temperature. In artificial cryptorchidism, for instance, epididymal spermatozoa show a rapid loss of function and soon degenerate (Glover, 1960; Cummins & Glover, 1970). In the mammalian testiconda, therefore, the question arises of how spermatogenesis proceeds at abdominal temperatures, and how spermatozoa can mature and survive in an epididymis that is permanently in the abdomen.

Harrison (1949) pointed out the unusual arterial architecture of scrotally situated testes, and there is evidence to suggest that this might be a disadvantage to the organs in adapting to increased temperature (Setchell & Waites, 1970). For this reason, the course and distribution of the testicular artery was examined in the present work, particular attention being paid to animals with abdominal testes.

Certain distinct changes are known to typify sperm maturation in the epididymis, and in mammals with scrotal testes, the tail of the epididymis is recognized as an area which is conducive to prolonged survival of mature spermatozoa. Although reference has been made to these events in the hyrax and the elephant (Glover, 1968; Glover & Sale, 1968), there is little information about them in African mammals generally. It was, therefore, decided to examine spermatozoa from different levels of the epididymis in the different species.

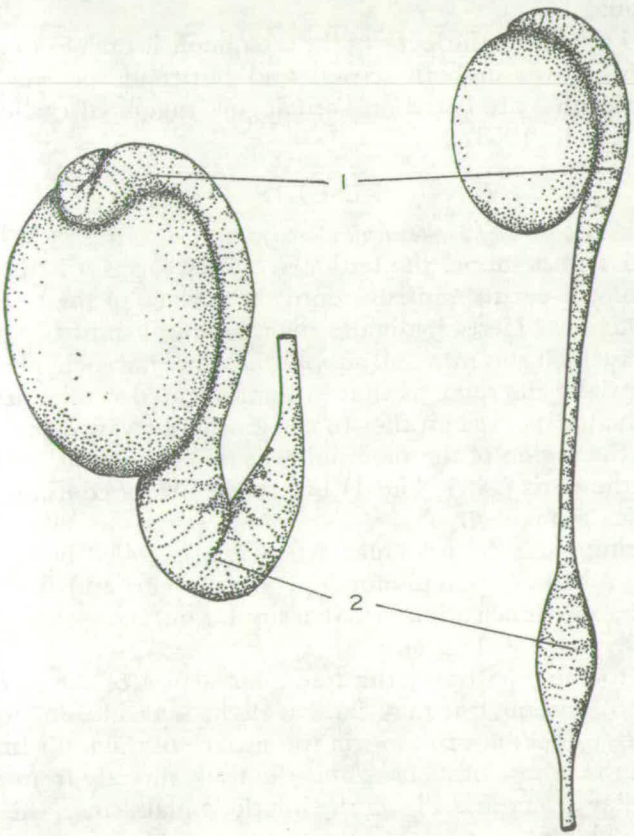
MATERIALS AND METHODS

Material was collected from two eland bulls, two steinbok, three giraffe, eight rock hyraxes and three elephants in Kenya. Further investigations were made on rock hyrax from the Transvaal, South Africa, and on one elephant shot on the banks of the Crocodile River bordering the Kruger National Park, near Komatipoort.

Examination of the testicular artery was carried out in each case according to the method of Harrison (1951), using an injection of radio-opaque material and the subsequent production of an arteriograph. Micropaque (barium sulphate, 100% w/v, Damancy & Co., Ltd) was used for direct injection into the testicular artery of larger species and into the thoracic or abdominal aorta of smaller species. The injection was always made slowly, either by using a simple record syringe or by gravity feed, according to circumstances. The giraffe was the only species in which it was found necessary to flush out the artery with saline before introducing the injection fluid. The particle size of the Micropaque is such that it does not pass through capillaries and thus it only fills the arterial side of the vasculature. After injection, the testes were removed and stored at a temperature of 0° C, or lower, before X-rays were taken.

For a thorough examination of material, it is difficult to perform all the required procedures on the same animal. Hence, spermatozoa were usually

taken from separate fresh specimens which were dissected out and examined as soon as possible after the death of the animal. Spermatozoa were collected by incising the epididymis at different levels (Text-fig. 1) and gently squeezing out the spermatozoa onto a microscope slide. The first sample was quickly examined under the microscope for motility. If the spermatozoa were immotile, the sample was slightly diluted with fructose Ringer phosphate solution (Mann, 1964). No attempt was made to quantify motility; a subjective assessment of



TEXT-FIG. 1. Homologous areas of the epididymis in a scrotal mammal (left) and a testicond mammal (right) from which spermatozoa were withdrawn for examination. 1 = caput epididymidis; 2 = cauda epididymidis.

'good', 'medium' or 'nil' was made. When this examination was complete, a further sample from the same area was taken, mixed with one drop of aqueous nigrosin eosin on a microscope slide, smeared onto the same slide and a duplicate smear made on another slide from the residual spermatozoa–stain mixture on the cover slip. Each was allowed to dry in air before being mounted in DPX. Later, 100 spermatozoa were counted on each slide for an estimate of morphological features. Further smears were made for staining with Papanicolaou's stain (Papanicolaou, 1942) for confirmation of the morphological picture.

Since it was not possible to test the fertilizing capacity of the spermatozoa, a morphological indicator of sperm maturation was used in addition to the observation of motility. The criterion taken was the migration of the cytoplasmic droplet to the end of the mid-piece (Lagerlöf, 1934; Merton, 1939). Contraction of the acrosome round the nucleus as seen in maturing rabbit spermatozoa (Bedford, 1965) was unreliable as a guide to sperm maturation in this instance because conditions were conducive to membrane damage and any abnormal swelling of the acrosomes would make quantitative interpretations difficult.

Measurements of sperm size were made with the use of an ordinary eyepiece micrometer gauge.

Text-figure 1 illustrates the levels of the mesonephric duct from which spermatozoa were withdrawn in both scrotal and testicond species. The levels in each type of mammal are based on homologues suggested earlier (see Glover, 1968; Glover & Sale, 1968).

RESULTS

The course and distribution of the testicular artery

In the eland and steinbok, the testicular artery forms a helix in the region of the pampiniform plexus and runs onto the surface of the testis underneath the tunica albuginea. Here, it divides dichotomously into two main arteries, the branches of which run into and supply the testis parenchyma. This arterial pattern is essentially the same as that in domesticated ruminants and is again to be seen in the testis of the giraffe. In the giraffe, however, the artery is more convoluted in the region of the pampiniform plexus and the coils extend onto the surface of the testis (Pl. 1, Fig. 1) before the artery continues in its course under the tunica albuginea.

In the jumping hare, the testicular artery is also coiled before reaching the testis. There is a distinct pampiniform plexus of veins and the artery courses tortuously under the tunica albuginea in a similar but more exaggerated fashion than that of the rat (Pl. 1, Fig. 2).

In contrast to these patterns, the testicular artery of the hyrax is straight (Harrison, 1958), although it may show a slight kink in some animals (Pl. 1, Fig. 3). The artery does not run far on the surface of the testis but divides into two major arteries whose branches enter the testis directly from one side only. The left testicular artery arises from the inferior renal artery, there being three arteries to each kidney.

The male rock hyrax is a seasonal breeder, and specimens were taken from both sexually active and sexually quiescent animals (Glover & Sale, 1968). Differences in the size of the testes between these two are often quite remarkable (Millar & Glover, 1970) and it was observed that, in sexually active animals, the testicular artery and its branches were much better filled with Micropaque than in sexually quiescent animals. It is thus evident that in sexual activity the blood vessels dilate and the testis becomes suffused with blood. Markedly distended veins filled with blood lend further support to this.

Characteristics of epididymal spermatozoa

A considerable difference was noted in the length and breadth of sperm heads

PLATE 1

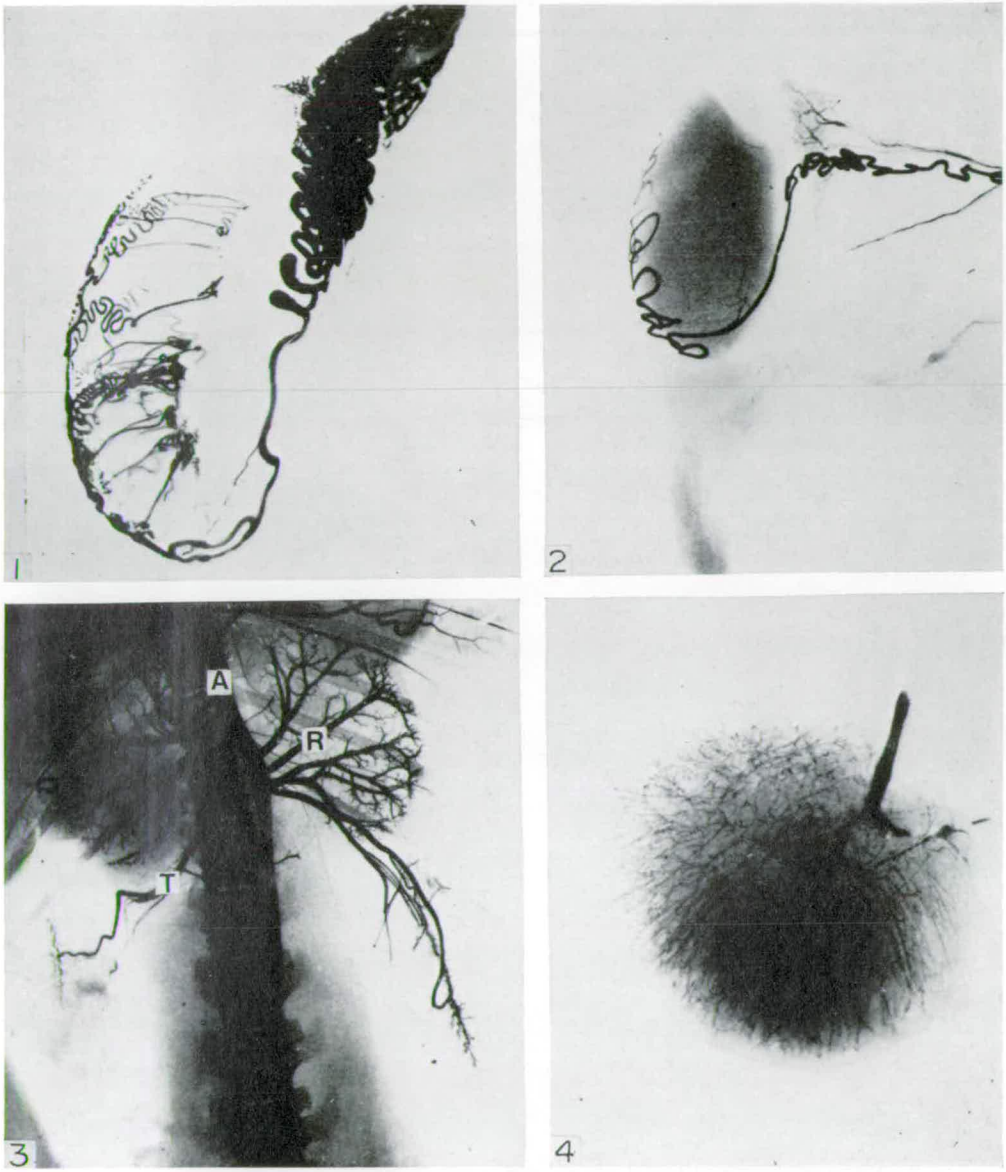


FIG. 1. Arteriograph showing the arterial supply to the testis of a giraffe.

FIG. 2. Arteriograph showing the arterial supply to the testis of a jumping hare.

FIG. 3. Arteriograph showing the arterial supply to the testis of a rock hyrax. A = aorta; R = renal arteries; T = testicular (internal spermatic) artery.

FIG. 4. Arteriograph showing the straight testicular artery of an elephant.

(*Facing p.* 48)

in the different species examined, as well as in the overall length of the spermatozoa, in the shape of sperm heads and in the length of the mid-pieces. Table 1 indicates that in all the species, spermatozoa acquire the capacity for motility as they move through the mesonephric duct, and that during this time the cytoplasmic droplet migrates towards the end of the mid-piece. During epididymal transit, the acrosome appears to contract down over the nucleus of the spermatozoon, but this could be an artifact and needs to be checked with the electron microscope. It is to be emphasized, however, that signs of maturation in the spermatozoa are just as evident when the excurrent ducts are in the abdominal and pelvic cavities as when they are in a scrotum.

Table 1. Incidence of spermatozoa with proximal cytoplasmic droplets and the motility of samples collected from two levels of the epididymis in four species of African mammals

Position of testes	*No. of animals*	*Species*	*Level of mesonephric duct**			
			1		2	
			Droplets (%) with ranges†	*Motility*	*Droplets (%) with ranges†*	*Motility*
Scrotal	3	Giraffe	82 (75 to 91)	Medium	1 (nil)	Good
	2	Eland	74 (69 to 78)	Medium	3 (1 to 5)	Good
Abdominal	1	Elephant‡	41 (39 to 43)	Nil	12 (nil)	Medium
	3	Hyrax (sexually active)	51 (42 to 60)	Nil	1 (0 to 2)	Good

* See Text-fig. 1.
† Mean percentage from duplicate smears.
‡ With fructose Ringer phosphate added (see text).

In all three elephants examined, there was little or no motility in sperm samples taken from any level of the mesonephric duct, but in one elephant it was found that sperm motility could be stimulated by the addition of fructose Ringer phosphate. When this was done, clear differences in the activity of immature and mature spermatozoa became apparent (see Table 1). In this context, therefore, the elephant would not seem to be basically different from other testicond mammals.

DISCUSSION

In this work, arteriographs suggest that convolution of the testicular artery has evolved in association with descent of the testes. Taxonomically, it is reasonable to believe that abdominal retention of the testes and a corresponding straight testicular artery represent a relatively primitive condition and that differences in the degree of convolution of the artery are simply the result of evolutionary divergence. Harrison (1949) and Harrison & Weiner (1949) contended that the length of the testicular artery is related to the degree of cooling of the incoming blood, although this might depend partly on the ambient temperature. In this connection, it is interesting that, in the giraffe, the artery is excessively

coiled, since the animal is a tall ruminant and is regularly exposed to the sun for long periods.

The testicular artery of the jumping hare is less coiled in the region of the pampiniform plexus, but coiling in the artery indicates that the testes in this species are essentially scrotal. When the animal is in a relaxed condition, the testes may be seen in the scrotum; it is only after death that they are almost always to be found in the abdomen. For this reason, they have erroneously been described as abdominal (Coe, 1969). It is possible, of course, that the jumping hare periodically withdraws its testes into the abdomen, but as with some lagomorphs which also show periodic withdrawal (Asdell, 1964), large inguinal canals and a coiled testicular artery may be taken as indicating that descent of the testes has at some time occurred during the animal's evolution.

There is nothing more than this to suggest that a coiled testicular artery, which continues its course superficially under the tunica albuginea, is related to the susceptibility of the germinal epithelium to fluctuations in temperature, though the straightness of the artery in testicond mammals (Pl. 1, Fig. 4) does suggest that abdominal testes might function rather differently from scrotally situated ones. The arterial design in the testes of testicond mammals would seem to allow a greater variation in blood flow, and in the hyrax signs of increased flow in the testes during sexual activity are striking. The increase in blood flow is interesting in relation to the animal's hormonal status at this time, when a high level of circulating gonadotrophins might be anticipated. In scrotally situated testes, there is evidence of considerable resistance to changes in blood flow (Glover, 1968; Setchell, 1970). It seems reasonable to conclude, therefore, that coiling of the artery in the region of the pampiniform plexus has been associated with testicular descent and that it might have been accompanied by specialization of spermatogenic tissue as an adaptation to the unusual environment in a scrotum.

Spermatogenesis in abdominal testes might not need an especially low temperature, and might even be stimulated when the temperature is increased. It has been shown that a high temperature stimulates spermatogenesis in the common frog, *Rana temporaria*, by accelerating the FSH activity of the pituitary (Van Oordt & Lofts, 1963) and the relationship of temperature and the hormonal control of the testes in mammals bears closer examination. By contrast, development of spermatogenesis in young birds appears to be speeded up at temperatures lower than the abdominal temperature, but the process does not seem to favour very low temperatures (Williams, 1958), and there is no direct evidence that spermatogenesis in birds is hypersensitive to elevated temperatures. The possibility could be tested, but the fact that body temperature in birds is higher than that of mammals suggests that there is considerable variation between vertebrate species in the sensitivity of their testes to high temperature. This is certainly worth bearing in mind in the case of man, where the testes may have become partially adapted to increased temperature.

There are marked differences in the dimensions of epididymal sperm heads in different species. It is probable, though, that the DNA content is relatively constant in mammalian spermatozoa and that apparent species variations in size mainly reflect differences in shape and thickness.

In all the species examined here, however, spermatozoa acquired the capacity for motility as they traversed the mesonephric duct, and the cytoplasmic droplet migrated to the end of the mid-piece. These changes were as obvious in testicond mammals as in those with scrotal testes, and are of interest in view of the fact that sperm survival in the epididymis is severely curtailed in artificially cryptorchid animals. It seems, therefore, that the function of the epididymis has been modified in conjunction with its descent into a scrotum, necessitating a relatively stable temperature for sperm maturation. This can be provided by the scrotum in most mammals, but in testicond mammals, thermoregulation is generally poor (Wislocki, 1949). In the rock hyrax, fluctuations in body temperature must be considerable (Taylor & Sale, 1969) as a result of the animal's normal habitat. Even if its body temperature is rather lower than that of most eutherian mammals, it would not adequately explain how its testes are able to function in the abdomen or its spermatozoa are able to mature in an abdominal epididymis when the ambient temperature is so variable. It certainly seems that the extreme sensitivity of testes to temperature fluctuation has occurred in association with the evolutionary development of homeothermy. Elephants, however, are out in the sun for most of the day and their body temperature has been shown to remain within average limits (Short, Mann & Hay, 1967). Nevertheless, more information on diurnal fluctuations in both body temperature and spermatogenesis in a wide variety of testicond mammals would be useful.

The survival of mature spermatozoa in the cauda epididymidis has been shown in rabbits to be dependent upon maintenance of the temperature within a fairly narrow range below that of the abdomen (Chang, 1943; Glover, 1958). In testicond mammals, however, the comparable region of the mesonephric duct lies relatively close to the surface of the body (Glover & Nicander, 1971). In the rock hyrax and the elephant, this part of the duct is distended and whiter than the rest of the duct, and it seems justifiable to regard it, like the cauda epididymidis, as a region of sperm storage. Spermatozoa probably move through it constantly, as they do through the cauda epididymidis, but the rate of transport must be much slower than in other regions, and the lumen is distended and packed with spermatozoa. In sexually active animals, the contents of the duct at this level are under pressure and tend to flatten the surrounding epithelium. These general features appear to typify the 'true testiconda', for they are also seen in a number of insectivores such as *Elephantulus*, *Petrodromus* and *Rhyncocyon* (T. D. Glover, personal observations). If this interpretation of homologous areas is correct, and the contributory evidence is now substantial, the ductus deferens in testicond mammals will be represented only by a short terminal length of the mesonephric duct.

From recent work (Glover & Nicander, 1971), it appears that, from a phylogenetic point of view, the sperm store in mammals has migrated towards the surface of the body and even left the confines of the abdomen before the testes. Testicular descent may well have been secondary to descent of the sperm store. At least, it would seem that during the course of evolution, the need for prolonged survival of spermatozoa in the mesonephric duct may have been a factor involved in the caudal migration of the gonads and their ducts.

ACKNOWLEDGMENTS

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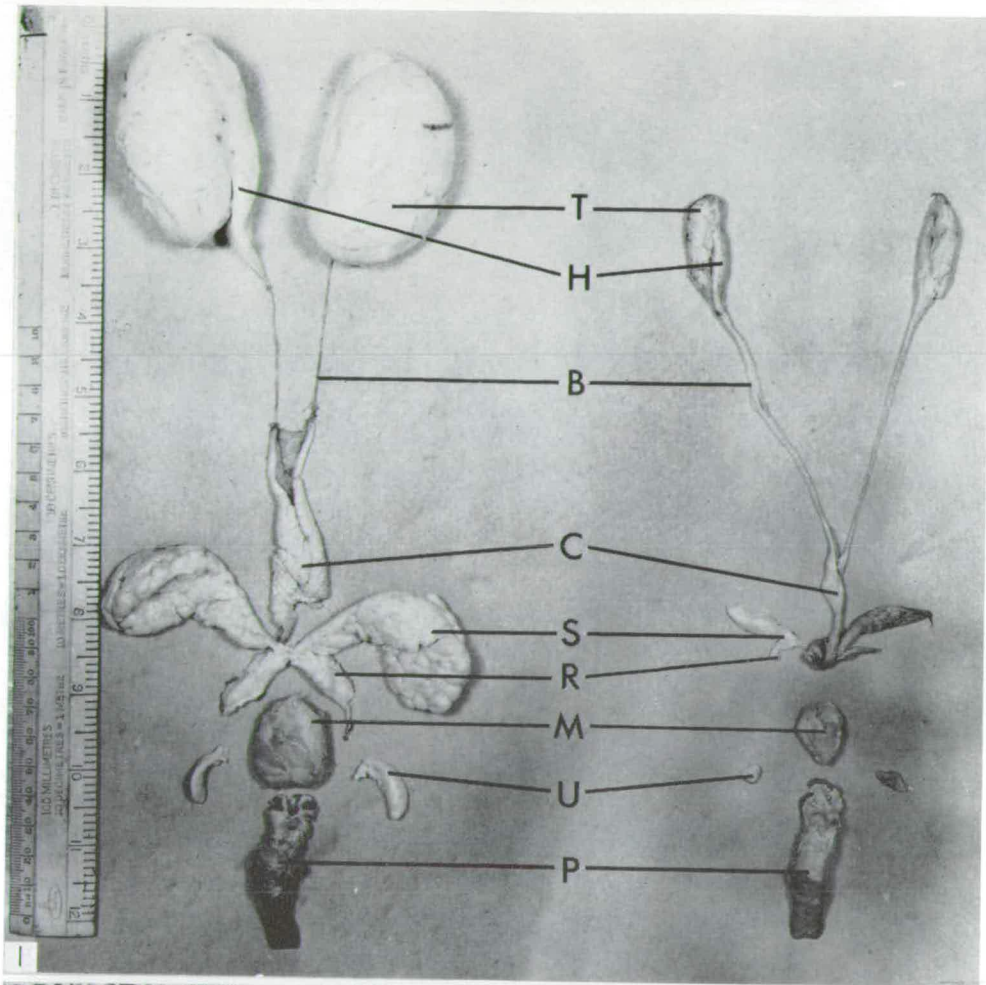
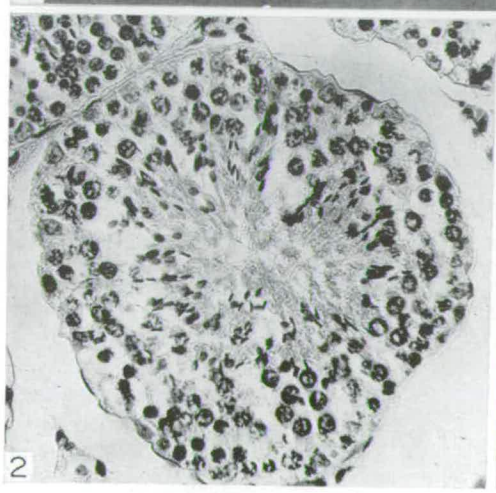
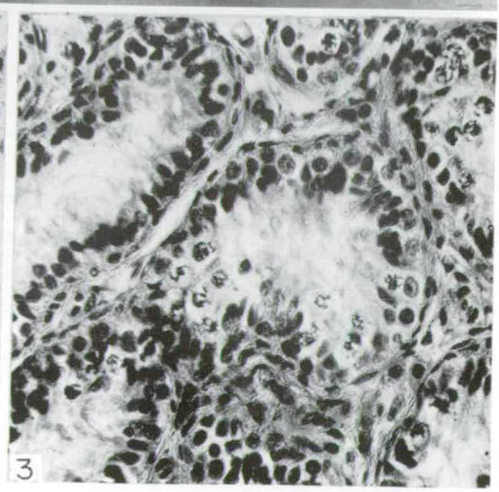
Seasonality in the male

(with special reference to the epididymis).

PLATE 1







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SEASONAL CHANGES IN THE REPRODUCTIVE TRACT OF THE MALE ROCK HYRAX, *PROCAVIA CAPENSIS*

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Glover & Sale (1968) described the occurrence of sexually active and sexually quiescent males in colonies of rock hyrax, *Procavia* and *Heterohyrax*, in East Africa, but previous attention has not been drawn to seasonal changes in the reproductive tract of the male South African dassie, *Procavia capensis*.

This work is part of a wider investigation of the ecology and reproduction of male dassies and the animals were collected in the Cape Province throughout the year. Examination of their reproductive tracts revealed that they are sexually quiescent between May and January. This is indicated by their having small testes, seminal vesicles and bulbo-urethral glands and by the total absence of spermatozoa in their reproductive tracts (Table 1). In February, however, dramatic changes in the size of the testes and accessory glands occur (Pl. 1, Fig. 1), and in some animals there may be as much as a twenty-fold increase in the weight of these organs. Concurrently, the production of spermatozoa is resumed and males exhibit copulatory behaviour.

Histological sections of the testes show that spermatogenesis is correspondingly inhibited between May and January. During this time, spermatozoa and spermatids are absent from the seminiferous tubules, spermatocytes are also absent or sparse and when present, often show abnormal nuclei like the remaining spermatogonia. Sertoli cells are prominent but the tubules themselves are small (Pl. 1, Fig. 3), and only a few dead and degenerating spermatozoa are seen in the epididymis. By contrast, testes collected during February and March show active germinal epithelium (Pl. 1, Fig. 2). The seminiferous tubules are often packed with spermatozoa and in our material, the number of spermatozoa

EXPLANATION OF PLATE 1

FIG. 1. Reproductive tract from a sexually active male hyrax (left) and from a sexually quiescent male hyrax (right). T = testis; H = head of epididymis (caput epididymidis); B = body of epididymis (corpus epididymidis); C = sperm store (cauda epididymidis); S = seminal vesicle; R = prostatic-type tissue akin to coagulating gland of rat (an extension of urethral prostatic tissue described by Glover & Sale (1968) and only observed so far in the South African dassie); U = bulbo-urethral (Cowper's) gland; P = penis. M = Bundle of penile muscles (see Glover & Sale, 1968).

FIG. 2. Testis from sexually active male hyrax. H & E, × 350.

FIG. 3. Testis from sexually quiescent male hyrax. H & E, ×350.

TABLE 1

MEAN NUMBER OF SPERMATOZOA IN SPERM STORE AND MEAN WEIGHTS OF TESTES AND ACCESSORY GLANDS DURING PERIODS OF SEXUAL QUIESCENCE AND ACTIVITY IN *Procavia capensis*

Time of year	*Wt of testes (g)*			*Combined wt of seminal vesicles and prostates (g)*			*Wt of bulbo-urethrals (g)*			*Total no. of sperm. in sperm store* ($\times 10^9$)		
	Mean	*Range*	*No. of animals*	*Mean*	*Range*	*No. of animals*	*Mean*	*Range*	*No. of animals*	*Mean*	*Range*	*No. of animals*
May to January	5·1	1·4 to 11·3	61	2·3	0·5 to 6·6	52	0·48	0·12 to 0·91	50	0·013	0·000 to 0·075	13
February and March	69·1	30·0 to 113·8	16	10·0	2·7 to 34·6	14	0·99	0·38 to 1·49	13	7·85	2·03 to 24·15	10

in the sperm store (cauda epididymidis) was $7{\cdot}85 \times 10^9$ (Table 1). At the beginning of April, the size and activity of the testes and accessory glands rapidly decline.

A few measurements of the diameter of seminiferous tubules showed that the increase in the size of the testes during the mating season is associated with a much larger diameter of the tubules. During the sexually active period, the mean diameter of the testes from eight animals was 3·2 cm and the mean diameter of the tubules in six of them was 382 μ. The mean diameter of testes in six sexually quiescent animals was only 1·7 cm and the mean tubule diameter in five was 111 μ. There is no obvious change in the bulk of interstitial tissue when sections are conventionally stained and examined under the light microscope, but Glover & Sale (1968) have demonstrated more sudanophilic cells in testes from sexually active animals.

Seasonal changes in the weight and activity of testes and accessory glands have been recorded in males of other mammals, but such striking increases in weight of testes during the mating season combined with complete cessation of spermatogenesis during the non-mating season are more prevalent in birds and reptiles. It is of interest that the hyrax has other features in common with these animals. They include a poorly developed capacity for general body thermoregulation, and the permanent retention of testes in the abdomen. It must be presumed, therefore, that this type of reproductive system, which is doubtless shared in principle by other mammalian species, is relatively primitive as far as eutherian mammals are concerned.

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J. Reprod. Fert., Suppl. 19 (1973), 203–220

REGULATION OF SEASONAL SEXUAL ACTIVITY IN AN ASCROTAL MAMMAL, THE ROCK HYRAX, *PROCAVIA CAPENSIS*

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Summary. The environmental cues regulating the reproductive rhythm of the male rock hyrax and the endocrine control of this rhythm are described.

There is a general increase in the activity of the pituitary of wild male hyraxes during the mating season which is ultimately responsible for the elevated androgenic and spermatogenic activity of the testes at this time. During the period of maximal testosterone production by the testes, the content of LH in the pituitary is markedly lower than during the early and late phases of testicular activity. This indication of the release of LH at a time when testosterone production is greatest, suggests that testosterone does not inhibit the release of LH to any significant extent during this phase of the reproductive rhythm. Experiments involving the administration of gonadotrophic hormone and stilboestrol supported these conclusions.

Collections of wild hyraxes in various study areas in South Africa showed that, as the latitude decreases, the copulatory period occurs later in the year and is longer in duration. This trend was not well correlated with annual changes in rainfall and temperature, suggesting that photoperiod is the environmental cue (proximate cause) for the mating season.

Experiments on captive hyraxes demonstrated that reduction of the photoperiod reactivates the testes, and it is suggested that a critical value of the rate of decrease in photoperiod is reached later at lower latitudes and corresponds to a similar trend in the timing of the mating season.

Several suggestions as to the ultimate cause of the mating season are also presented, and the relative importance of each is discussed.

INTRODUCTION

The susceptibility of mammalian testes to elevated temperature has long been recognized, and Moore (1924a, b, 1926) and Moore & Quick (1923, 1924) established the thermoregulatory function of the scrotum. It is evident that the

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rôle of the scrotum is to maintain the testes at a temperature that is suitable for spermatogenesis, and that this is lower than the temperature of the abdomen. This holds true for most mammalian species and thus poses the interesting question as to how spermatogenesis can proceed in mammals that have intra-abdominal testes. Moreover, it has been shown that, in a scrotal mammal, spermatozoa in the epididymis are sensitive to slight increases in temperature (Glover, 1960, 1962; Cummins & Glover, 1970), so that, in testicond mammals, the maturation and storage of epididymal spermatozoa, if it occurs in these species, must also take place at abdominal temperature.

The rock hyrax, *Procavia capensis*, is particularly interesting because in non-equatorial regions such as South Africa the male, in addition to having intra-abdominal testes, also has a very restricted period of sexual activity (Millar & Glover, 1970; Millar, 1971). Furthermore, the rock hyrax exhibits poor physiological thermoregulation and experiences considerable diurnal and annual variations in body temperature (Taylor & Sale, 1969; Sale, 1970; Bartholomew & Rainy, 1971; Millar, 1972). It seemed possible, therefore, that there might be some relation between changes in annual environmental temperature and the reproductive rhythm.

In South Africa, earlier observations on the mating season of the hyrax in relation to annual climatic changes suggested that photoperiod is the proximate cause (environmental cue) regulating the timing of the mating season (Millar, 1971). This is supported by marked differences in breeding patterns in different geographical locations and in captivity (Mendelssohn, 1965; Glover & Sale, 1968; Griner, 1968; Sale, 1969). The ultimate cause, however, remained unresolved.

This communication reports on some further investigations into the proximate cause of the mating season and upon possible ultimate causes, including environmental temperature.

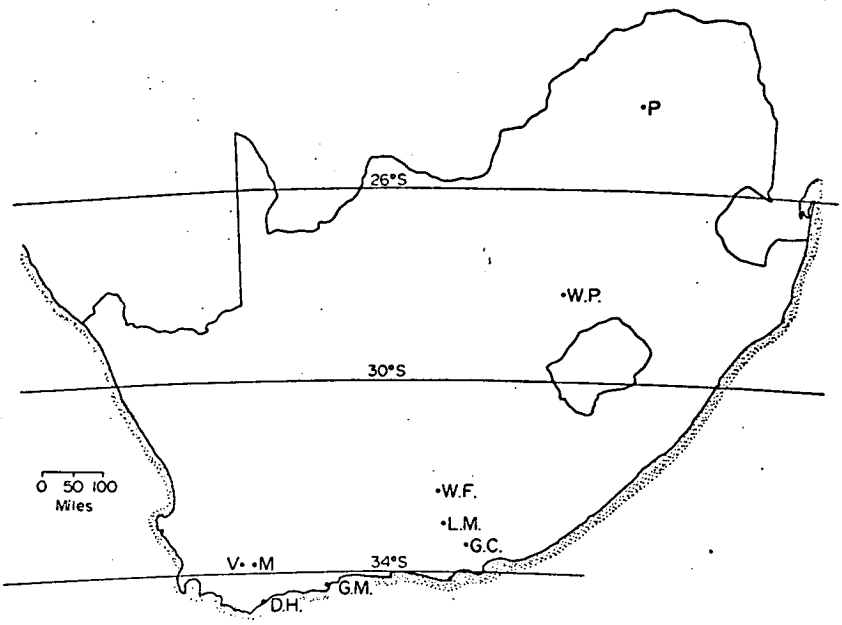
MATERIALS AND METHODS

Endocrine control of the reproductive rhythm

Field work. In the main study area, Lake Mentz, (Text-fig. 1) at least nine adult male hyraxes were collected every month throughout the year. The animals were dissected immediately after death and pieces of the left testis of each animal were fixed in Bouin's fluid and routinely processed for histological examination. The mean diameter of the seminiferous tubules was calculated from ten measurements with a calibrated eyepiece graticule.

The entire right testis was finely chopped into 2·5% NaOH, stored at −20° C, extracted and assayed for testosterone content by means of a standard radioimmunoassay using an antibody raised to BSA linked to the 3 keto group of testosterone (developed by P. Rowe, unpublished data).

The pituitary glands of four males in each collection during the mating season were dissected and stored at −20° C. Later, they were pooled, homogenized in physiological saline and assayed for LH activity, using the ovarian ascorbic acid depletion bioassay (Parlow, 1958) and for FSH by the method of Steelman & Pohley (1953).



TEXT-FIG. 1. Hyrax study areas in South Africa. D.H., De Hoop; G.M., Gouritzriviermond; G.C., Glenconnor; L.M., Lake Mentz; M, Montagu; P, Percy Fyfe game reserve; V, Vrolijkheid; W.F., Waterfall; W.P., Willem Pretorius game reserve.

Pituitaries from other males were fixed in formol sublimate, embedded in paraffin wax and sectioned and stained with Alcian Blue, PAS and Orange G in order to identify gonadotroph cells in the pars distalis.

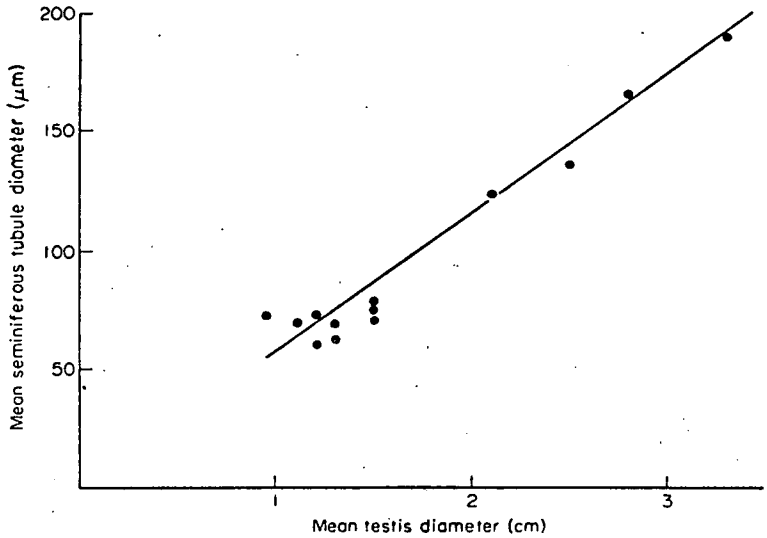
Table 1. Effects of subcutaneously injected PMSG on sexually quiescent males

Animal no.	*Treatment begun*	*Dose (i.u.)*	*Duration (weeks)*	*Total dose (i.u.)*	*Result*
PMSG 1	2 weeks before testes of controls enlarged	75/2 days	1½	375	Animal ill; no response by time of death
PMSG 2	As above	75/2 days	2	525	As above
PMSG 3	As above	75/day	1½	450	As above
PMSG 4	As above	75/day 150/week	2 23	1050 3450	No initial response; testes enlarged at same time as in controls
PMSG 5	2 weeks after testes regressed	150/week	5	750	Testes enlarged 1 week after first injection
PMSG 6	9 weeks after testes regressed	500/week*	11	5500	As above
PMSG 7	12 weeks after testes regressed	200/week 500/week	1 3	200 1500	Testes enlarged 1 week after 200 i.u. PMSG inject.

* Also received 1500 i.u. HCG/week for the last 3 weeks of treatment.

Hormone treatments. As indicated in Table 1, seven sexually quiescent adult male hyraxes were injected with various doses of PMSG (Folligon, Organon Ltd) starting at different stages of the reproductive cycle. One sexually quiescent male was force-fed with 100 mg stilboestrol in arachis oil once a week for 12 weeks, starting just before the onset of the mating season. Four untreated males served as controls. The animals were housed in individual cages at 25° C (± 5° C) and illuminated by normal daylight.

The degree of reproductive activity in each animal was determined weekly by palpating both testes through the abdominal wall and measuring the short diameter of each with a vernier caliper. This provides a good index of testicular activity, since there is a direct relationship between the mean short diameter of the testes and the mean diameter of the seminiferous tubules (Text-fig. 2).



TEXT-FIG. 2. Correlation between mean seminiferous tubule diameter and mean short diameter of the testes of hyraxes collected each month at Lake Mentz.

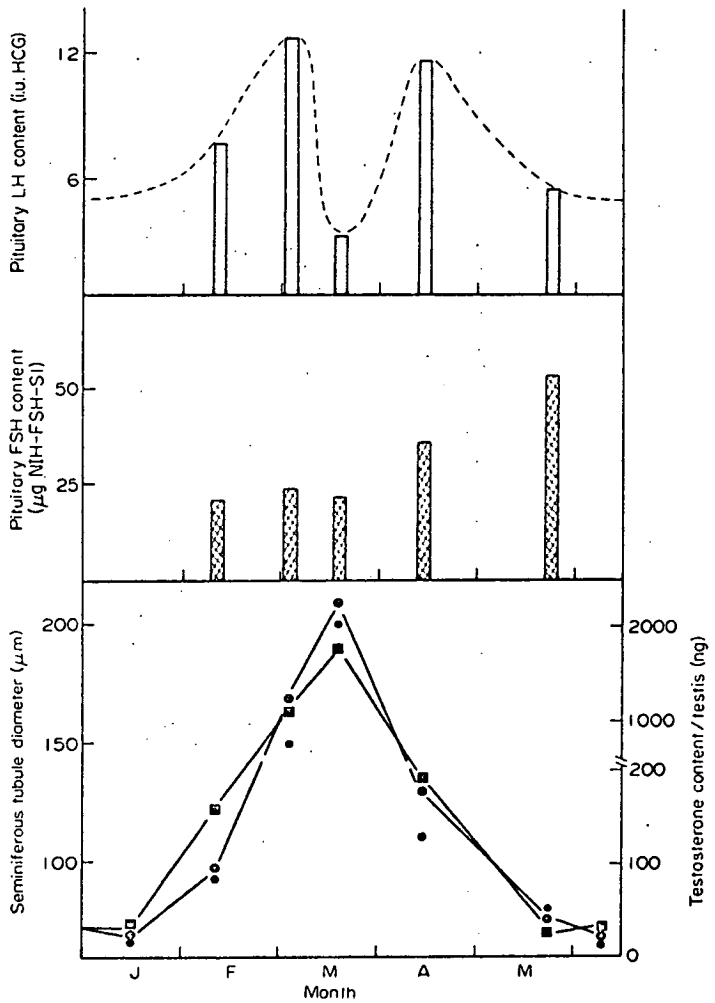
Environmental regulation of the reproductive rhythm

Field work. Random collections of male and female rock hyraxes were made in various study areas in southern Africa (Text-fig. 1). Testes and fetuses were removed and weighed, and dates of conception were calculated by using a fetal growth curve (see Millar, 1971).

The field data, examined in relation to annual changes in temperature, rainfall and photoperiod in the respective collection areas, suggested that photoperiod was the primary environmental cue to reproductive activity in the males. This possibility was tested experimentally.

Experiments. Initially a group of one prepubertal and three adult male hyraxes, all of which were expected to become sexually active during the ensuing mating season, were kept in complete darkness except for 10 min each day when the cage was cleaned and the animals fed, while a second group, consisting of two

adult males, was maintained in continuous light provided by a single 100-W lamp inside the cage. The experiment began some 6 weeks before the expected time of testicular regeneration and continued until after the testes of four control animals had regressed at the end of the season.



TEXT-FIG. 3. Seasonal changes in mean pituitary content of LH and FSH in relation to changes in mean seminiferous tubule diameter (□) and mean testis content of testosterone (●). Small circles show standard error of the mean testosterone content. 1 i.u. HCG approximately equal to 2 μg NIH-LH-S1.

On returning to Liverpool, a single available adult male was subjected to an increasing photoperiod, followed by a rapidly decreasing one (see Text-fig. 9). Experimental treatment commenced 5 weeks after the testes of this animal had regressed at the end of the normal mating season. Throughout the experiment, the temperature was maintained at 25°C.

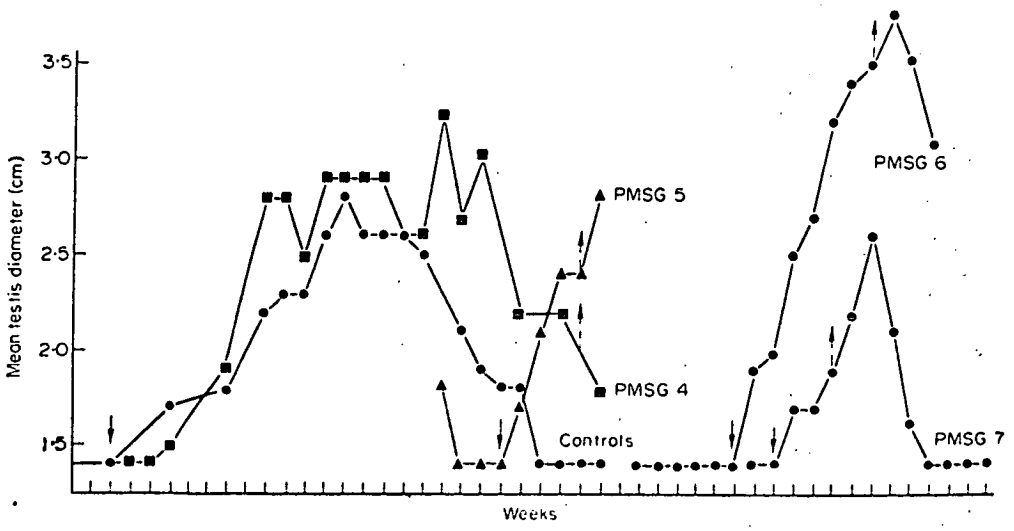
RESULTS

Endocrine control of the reproductive rhythm

Seasonal endocrine changes. In Text-fig. 3, seasonal changes in the pituitary content of FSH and LH are shown in relation to changes in mean seminiferous tubule diameter and testosterone content of the testes. Although the overall pattern of seasonal changes in the pituitary content of LH was similar to that of the changes in the testis content of testosterone, there was a marked decrease in LH content of the pituitaries at the peak of testis activity when testosterone levels were at their highest. There was also some indication of a slight depletion in pituitary content of FSH at this time, and a large increase in the content of FSH at the very end of the mating season, when seminiferous tubule diameter had declined to quiescent values and spermatogenesis had ceased.

Histological studies of the anterior pituitary of sexually quiescent males revealed that very few gonadotroph cells were present, while in sexually active males these cells were common and the amount of cytoplasm in the cells was extensive and generally filled with PAS-positive granules. However, at the peak of testicular activity, although gonadotroph cells were numerous, the cytoplasm was degranulated and foamy in appearance and somewhat similar to that seen in 'castration cells'. In the next collection of pituitaries, which was made when gonadal activity was declining, there was some regranulation of gonadotrophs, but, in the subsequent samples, taken when the testes were once again quiescent, very few active gonadotrophs were to be seen.

Hormone treatments. In the four male hyraxes that had received PMSG before the normal time of testicular regeneration, the testes failed to respond to the treatment (Table 1). Three of the animals died of pneumonia before showing any signs of enlargement of the testes, and the testes of the remaining male (No. 4) increased in size at the same time as those of untreated control animals

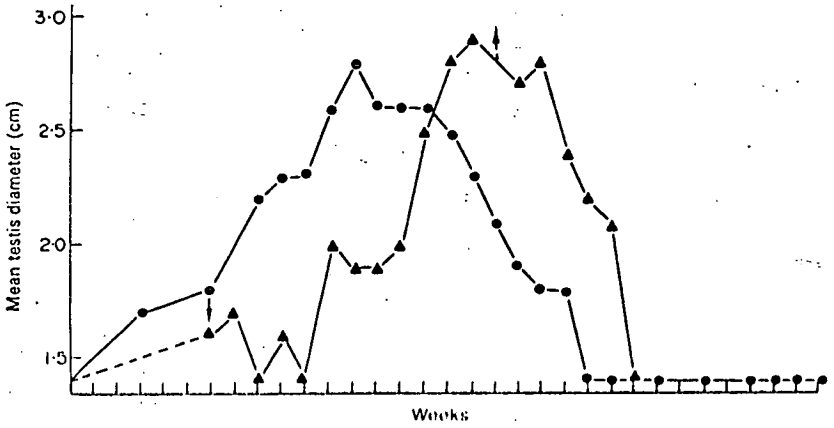


TEXT-FIG. 4. Effect of injecting PMSG into sexually quiescent male hyraxes. Dosages shown in Table 1. ↓, Start of treatment, ↑, withdrawal of treatment.

(Text-fig. 4). Continued administration of PMSG to this animal after the testes of control animals had regressed, maintained the gonads in an active state, but there appeared to be some reduction in size towards the end of the treatment period (Text-fig. 4).

The three animals (Nos. 5, 6 and 7) which received PMSG after the mating season, responded quickly; their testes increased in size within 1 week (Text-fig. 4), continued to respond to hormone treatment and regressed when it was withdrawn.

The testes of the hyrax which received weekly oral doses of stilboestrol remained small when those of control animals began to enlarge (Text-fig. 5). However, although treatment was continued, this inhibition was eventually overcome and, when the testes of control animals had reached maximum size, those of the stilboestrol-treated hyrax began to enlarge. The testes reached a peak of development 5 weeks later, and regressed during the following 7 weeks.



Text-fig. 5. Effect of oral administration of stilboestrol to a male hyrax during the mating season. ●, Control hyrax, ▲, stilboestrol-treated hyrax. ↓, start of treatment; ↑, withdrawal of treatment.

Environmental regulation of the reproductive rhythm

Field work. Random collections of the reproductive tracts of male hyraxes during the year in the various study areas indicated that the sexually active period occurred later and lasted longer at progressively lower latitudes (Table 2). This trend is not well correlated with annual changes in temperature and rainfall in the study areas.

A more comprehensive collection of male hyraxes was made in two of the areas (Lake Mentz and the Willem Pretorius game reserve) and the seasonal changes in weight of the testes are shown in relation to annual changes in temperature, rainfall and photoperiod in Text-fig. 6. Resumption of testicular activity in both areas occurred just after the summer solstice, but was not clearly correlated with the pattern of annual rainfall in the two areas. Rainfall varied too widely from year to year to be considered as the proximate cause of such a consistently timed mating season. Although, at first sight, the mating season would seem to be

Table 2. The period of testicular activity of hyraxes in the various study areas in relation to the prevailing climate

Area	*Sexually active period*	*Rainfall (mm)*	*Climate*
De Hoop 34° 35′S	February	All seasons, 450	Moderate
Montagu 33° 50′S	February/March	Winter, 300	Cold winters
Vrolijkheid 33° 50′S	February/March	Winter, 300	Cold winters
Glenconnor 33° 20′S	March/April	Sporadic, autumn maximum 250	Cold steppe
Lake Mentz 32° 55′S	March/April	Sporadic, autumn maximum 350	Cold steppe
Waterfall 32° 30′S	March/April	Sporadic, autumn maximum 350	Cold steppe
Willem Pretorius 28° 2′S	March, April/May	Autumn, Summer 600	Warm temperate
Percy Fyfe 24° 1′S	May/June, July	Summer, 450	Hot steppe

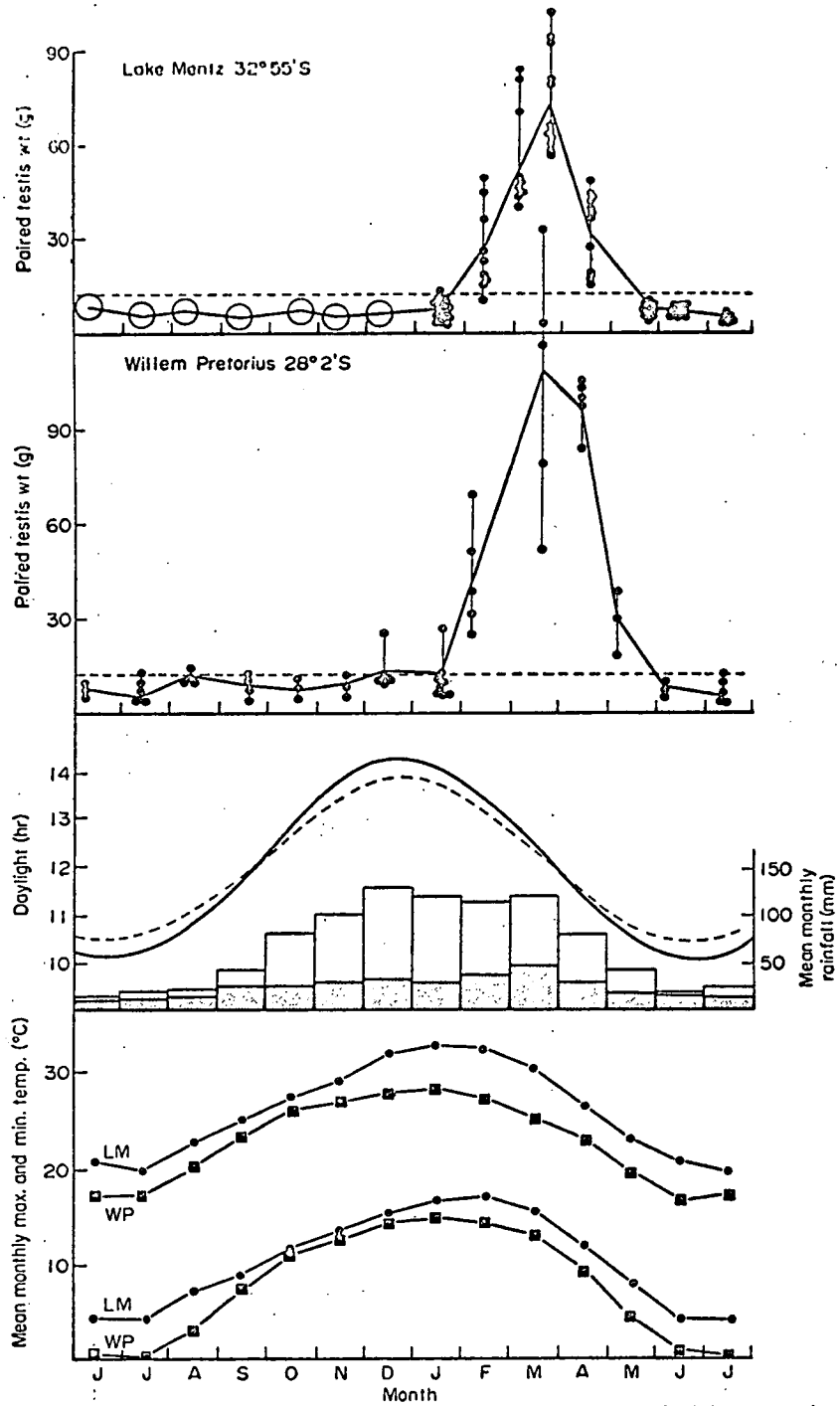
correlated with temperature, this is unlikely because significant changes in temperature only occur in March, when the testes have already become active.

Text-figure 6 also shows that the mating season in the Willem Pretorius game reserve (28° 2′S) lasts longer than at Lake Mentz (32° 55′S). In both areas, testicular weight first increased in February and reached a peak in March but, whereas the testes of hyraxes in the Willem Pretorius game reserve remained active during April, there was a rapid regression of the testes of hyraxes at Lake Mentz at this time (Text-fig. 6). Even in May, some animals with fairly active testes were still to be found in the Willem Pretorius game reserve. There is also some indication that the enlargement and regression of the testes of individual males is less well synchronized in hyraxes at Willem Pretorius, because the variation in testicular weights in any particular collection tended to be greater than in animals collected at Lake Mentz.

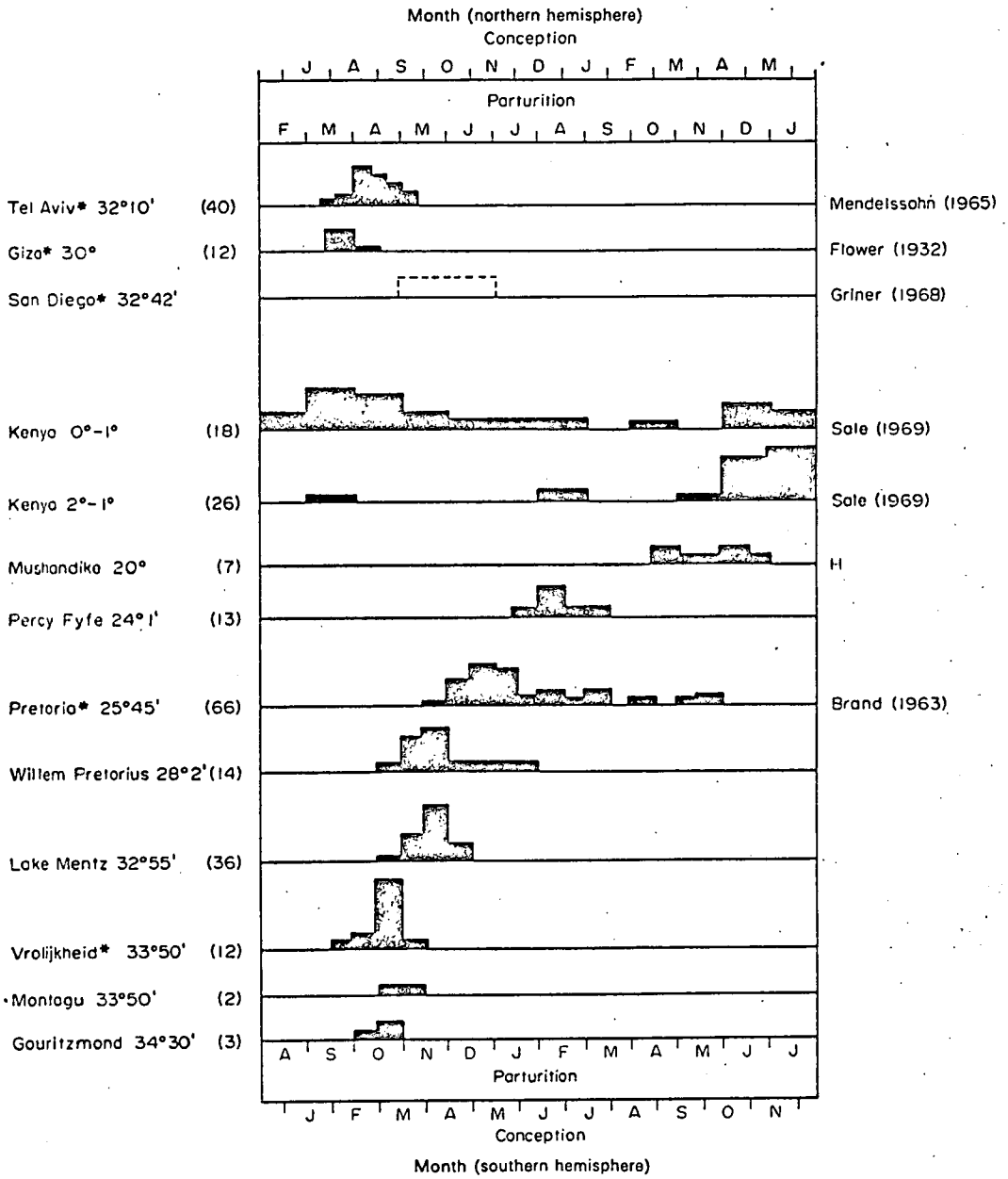
More comprehensive data on the timing of the mating season were calculated from the frequency distribution of births in the various areas under study and from births of hyrax that are quoted in the literature. The frequency distribution of births and conceptions, in areas in the latitude range 34°S to 33°N (Text-fig. 7), clearly demonstrates that the mating season occurs later, and generally lasts longer, in lower latitudes until, at the equator, there is a tendency for mating to take place throughout the year. In addition, the mating season in northern latitudes is approximately 6 months out of phase with that in corresponding southern latitudes (cf. San Diego, 32° 42′N, and Lake Mentz, 32° 55′S).

Experimental alteration of photoperiod

Changes in the size of the testes of male hyraxes exposed to continuous light occurred at precisely the same time as in control animals kept in normal daylight (Text-fig. 8a and b), but the response of hyraxes subjected to constant darkness was variable. The prepubertal animal, i.e. 17 months of age at the start of the experiment, failed to show any marked testicular development (No. 365,

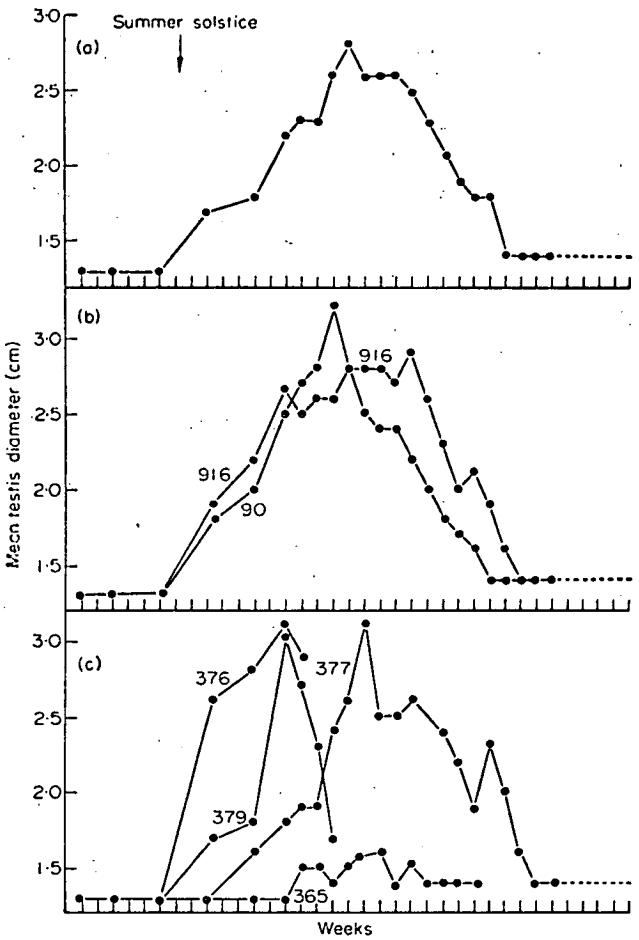


TEXT-FIG. 6. Reproductive rhythm of male hyraxes at Lake Mentz and Willem Pretorius game reserve in relation to annual climatic changes. Daylight: ——, Lake Mentz; – – –, Willem Pretorius. Rainfall: shaded columns, Lake Mentz; open columns, Willem Pretorius. Temperature: ○, Lake Mentz; ▣, Willem Pretorius. Large circles enclose range of testis weights. No signs of spermatogenesis are seen in testes in which the weight falls below the broken line.



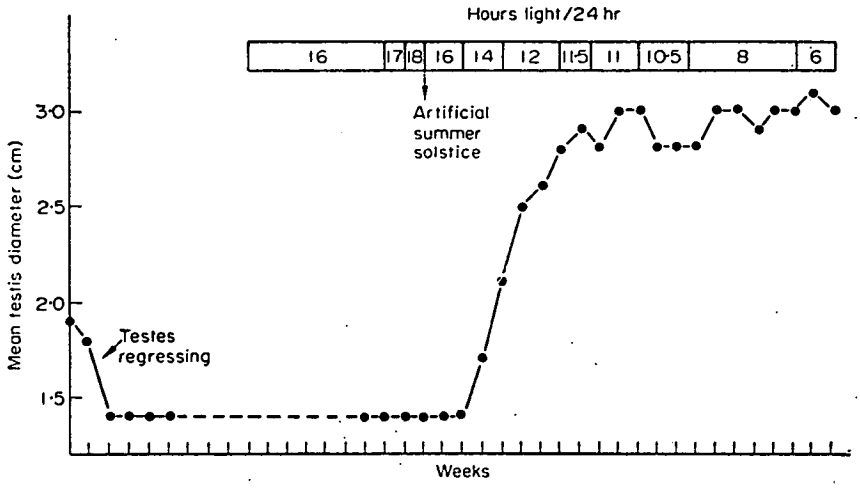
TEXT-FIG. 7. Frequency distribution of births and conceptions of hyraxes (*Procavia*) living at different latitudes. Number of litters shown in brackets; broken line for San Diego encloses the extremes of recorded births since data on the number of births in specific weeks were not available. Number of litters shown in parentheses. *, Captive colonies. H, *Heterohyrax*.

Text-fig. 8c). Males of 29 months of age or older (Nos. 376, 377 and 379), which had been sexually active in previous seasons, exhibited the normal reproductive rhythm, although there was a tendency for the testes to enlarge and reach maximum size slightly earlier (Text-fig. 8c).



TEXT-FIG. 8. Effects of continuous light and continuous darkness on the reproductive rhythm of the male hyrax. Changes in testis diameter in (a) four control hyraxes, (b) hyraxes in continuous light and (c) hyraxes in continuous darkness.

The testes of the hyrax which was subjected to an increasing photoperiod followed by a rapidly decreasing photoperiod, showed the first signs of enlargement 3 weeks after the first decrease in photoperiod (Text-fig. 9). Once the testes had reached maximum size, continued rapid decreases in photoperiod maintained them in an active state for a period far exceeding the normal duration of testicular activity.



TEXT-FIG. 9. Induction of out-of-season testicular regeneration in a hyrax subjected to increasing, followed by decreasing, photoperiod.

DISCUSSION

The rôle of the anterior pituitary in the control of the seasonal reproductive rhythm in the male rock hyrax is suggested by the higher incidence of gonadotroph cells and a higher content of LH in pituitaries during the mating season. Moreover, the immediate regeneration of the testes of hyraxes injected with PMSG after the end of the mating season demonstrates that the natural decline in testicular activity is a direct consequence of a reduction in circulating gonadotrophin and is not due to exhaustion or fatigue of the gonads or to their lack of responsiveness to gonadotrophins.

At the peak of testis development, when testosterone levels were at a maximum, the content of LH in the pituitary was at its lowest level. This depletion of LH in the pituitary most probably reflects hyper-release of the hormone, and histological findings also indicated that gonadotrophin secretion was high at this time because, although many active gonadotrophs were to be found, the cytoplasm of the cells was markedly degranulated. Thus, when testosterone production is at its peak, it would appear that feedback sites are relatively insensitive to its inhibitory effects and that LH release from the pituitary is relatively unrestricted. This is supported by the finding that even stilboestrol cannot prevent testis regeneration at this time. At the beginning of the mating season, however, when testosterone levels are much lower, the pituitary content of LH is high. This could be interpreted as being a consequence of increased synthesis of LH, and in addition, of a restricted LH release resulting from testosterone exerting its inhibitory effect at a lower threshold. Similarly, the increase in pituitary content of LH towards the end of the season indicates that synthesis of the hormone continues, and that the inhibition of LH release by testosterone is again operating at a relatively low threshold.

A similar situation seems to occur in the roebuck and red deer, because Short

& Mann (1966) and Lincoln, Youngson & Short (1970) reported that testicular testosterone levels are highest during the rut, while Brüggemann, Adam & Karg (1965) observed that the concentration of LH in the pituitary was markedly lowered during this phase. Again, although there is an overall increase in the LH content of the pituitary in rams during the mating season, there is a similar depression of LH levels at the peak of testicular activity (Thibault, Courot, Martinet, Mauléon, du Mesnil du Buisson, Ortavant, Pelletier & Signoret, 1966).

From the present findings in the hyrax, as well as from those in the roebuck and red deer, it is suggested that two main features of endocrine function control seasonal changes in testosterone production, namely, a general increase in synthesis of LH during the mating season, and an elevation of the threshold at which testosterone inhibits the release of LH during the peak of testicular activity. It seems probable that both the increase in pituitary synthesis of LH and the change in the threshold at which testosterone inhibits its release are controlled by higher neuroendocrine changes which are ultimately regulated by environmental cues.

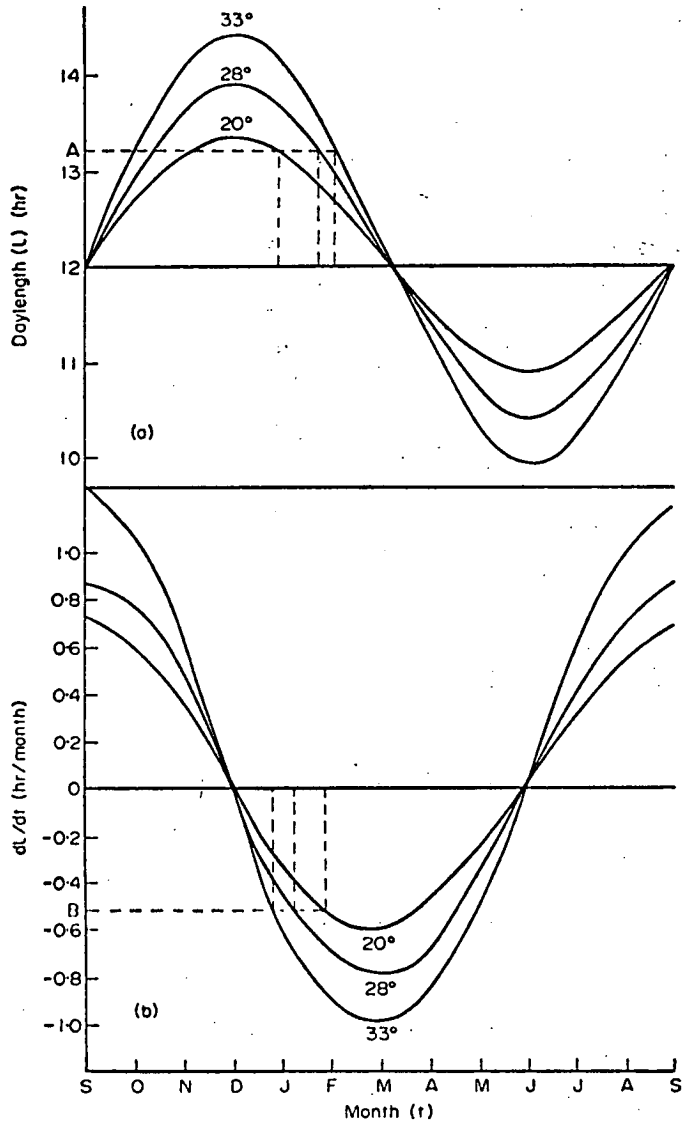
A dearth of fundamental knowledge about the control of both the production and release of FSH in male mammals makes it difficult to interpret the significance of seasonal changes in the pituitary content of FSH in the hyrax. For example, it is known that testosterone has little inhibitory effect on the release of FSH by the pituitary (see Davidson, 1966). Furthermore, the precise rôles of LH and FSH in the male are still uncertain, and since LH can maintain all known aspects of testicular function in hypophysectomized male rats (Simpson, 1959; Lostroh, 1969), the function of FSH in relation to spermatogenesis may lie mainly in its synergizing the effects of LH (Woods & Simpson, 1961). The present findings in the hyrax tend to support this, because levels of FSH in the pituitary are relatively unchanged until after the peak of testis activity, and this indicates a constant rate of release of the hormone. The only significant change seen was an increased pituitary content of FSH at the very end of the mating season which most probably reflects an inhibition of release of the gonadotrophin.

The timing of the mating season in relation to annual climatic changes in the various study areas strongly suggests that photoperiod is the environmental cue (or proximate cause) in the regulation of the reproductive rhythm of the male hyrax. Annual variation in temperature and rainfall in the different study areas is so great that they cannot be regarded as primary proximate causes, although they may well have a modifying influence. The fact that the mating season occurs soon after the summer solstice further suggests that it does so in response to decreasing photoperiod, as in other 'short-day' breeders such as sheep (Yeates, 1949; Ortavant & Thibault, 1956), red deer (Jaczewski, 1954) and sika deer (Goss, 1969a, b). The stimulation and extended maintenance of testis activity in the hyrax exposed to decreasing photoperiod further confirms this.

The fact that the testes of males maintained in continuous light became active at the normal time of the year, suggests that they had acquired an entrained annual rhythm which, in the absence of appropriate changes in light, still continued. Goss (1969a, b; Goss & Rosen, 1973) found a similar effect in adult

sika deer stags. It is significant that, in our work, the only prepubertal male hyrax, which was due to become sexually active for the first time in the ensuing season, failed to do so when exposed to continuous darkness.

With decreasing latitude, the mating season of the male hyrax occurs later. Although this may be due to variations in responsiveness of different populations to photoperiod, the trend strongly suggests that some controlling feature of photoperiod occurs later with decreasing latitude. For this reason, two functions of photoperiod, namely, absolute length of photoperiod (l), and the first differential of 'l' with respect to time (dl/dt) or rate of change of photoperiod,



TEXT-FIG. 10. Annual changes in (a) photoperiod, and (b) rate of change in photoperiod (dl/dt) at latitudes of three study areas. Points on t axis are at the 21st of each month.

are shown in Text-fig. 10 for three arbitrary latitudes in southern Africa. Text-figure 10(a) which represents annual changes in photoperiod clearly demonstrates that an absolute value of decreased photoperiod, e.g. 'A', cannot be the main factor determining the onset of testicular activity, since such a critical value would be reached earlier at lower latitudes instead of later. Thus, the rate of decrease in photoperiod is more likely to be the causal factor, and Text-fig. 10(b) shows that a critical rate of decrease in photoperiod, e.g. 'B', is, in fact, reached later at lower latitudes.

The greater variation in weight of testes in each collection at the Willem Pretorius game reserve, when compared to that of testes collected from Lake Mentz, suggests that the tendency for the mating season to last longer in lower latitudes is a consequence of a greater asynchrony in the reproductive rhythms of individual males, rather than to prolonged sexual activity in all of them. This may possibly be attributed to the fact that the amplitude of annual changes in photoperiod is always less at lower latitudes, and that animals living in lower latitudes may not be capable of perceiving changes in photoperiod with the same precision as hyraxes in higher latitudes. At the equator, although a few sexually active males are to be found throughout the year, the majority are in a sexually quiescent state, with small flaccid testes and only a few disintegrated spermatozoa (Glover & Sale, 1968). The presence of the latter implies that these were males which had only recently become sexually quiescent, since in South African hyrax such spermatozoa are only seen at the end of the mating season (R. P. Millar, unpublished data). Thus, even at the equator, where births occur throughout the year, individual males still appear to experience phases of sexual activity and quiescence.

Although the foregoing conclusions on the photoperiodic regulation of the reproductive rhythm in the male hyrax correspond broadly with field data on the mating season at different latitudes, certain shortcomings are apparent.

For instance, the timing of the mating season of the hyrax at various latitudes (Text-fig. 7) does not closely correspond to the times at which a critical value in the rate of decrease in photoperiod is reached (Text-fig. 10b). Furthermore, in the lower latitudes, such as Mushandike in Rhodesia (20°S), the mating season occurs from September to November when photoperiod has already started to increase. It has been suggested, therefore, that increasing temperature rather than photoperiod might be the proximate cause of mating seasons in the hyrax and other indigenous species in these lower latitudes (P. Wright, personal communication). In this event, it may be that photoperiod regulates the mating season in higher latitudes, and that in the lower latitudes nearer to the equator, where annual changes in photoperiod are not so great, temperature or other factors are of greater importance as environmental cues.

Since the mating season of the hyrax in South Africa occurs later with decreasing latitude, environmental factors which constitute the ultimate cause of the mating season would be expected to show a corresponding trend. The improved nutritional value of vegetation during rainy seasons often presents optimal conditions for the birth, development and suckling of young in many species and could, therefore, be the ultimate cause of the mating season in the hyrax. Yet the timing of parturition of hyrax, in the areas studied in South Africa, does

not seem to be correlated with the vast differences in annual rainfall patterns in the areas. For example, in the winter rainfall areas in the southern Cape, parturition occurs in October while, in summer rainfall areas, it takes place only a few months later.

Seasonal changes in temperature are possibly an important factor to the hyrax because it is a thermolabile animal and exhibits poor physiological regulation of body temperature. Indeed, deaths from pneumonia (particularly of young in captive colonies) are common during the winter months (Millar, 1972). However, it is again difficult to imagine that a trend in optimal temperatures with decreasing latitude coincides with parturition, because in the generally cooler most southerly areas of South Africa, the young are born in early summer, before the warmest time of the year, while further north they tend to be born in midsummer at the hottest time of the year. Thus, if the timing of the mating season has been selected to ensure that the young are born at the most favourable time of the year for their birth and early development, such seasonal optimal conditions must be inherent in a subtle combination of several environmental factors which is not readily apparent.

Because the male hyrax has intra-abdominal testes and is thermolabile, it is just possible that elevated temperatures during the summer might be deleterious to spermatogenesis and the survival of spermatozoa. In attempting to explain the ability of testicond mammals to produce spermatozoa at body temperature, Wislocki (1933) suggested that body temperature is lower in these mammals. But, clearly, this is not always the case, because in both the elephant (Wislocki, 1933; Benedict & Lee, 1936; Buss & Wallner, 1965; Short, Mann & Hay, 1967) and the hyrax (Taylor & Sale, 1969; Sale, 1970; Bartholomew & Rainy, 1971) body temperature can be as high as, if not higher than that in many scrotal mammals (36 to 37·5°C). Moreover, body temperature is probably indicative of testicular temperature in testicond mammals because measurements in a sexually active hyrax showed that there is little difference between rectal and testicular temperature (R. P. Millar, unpublished data). This suggests that, in an animal such as the hyrax, the temperature at which spermatogenesis is adversely affected is higher than in mammals with a scrotum. This seems quite reasonable in view of the fact that birds successfully produce spermatozoa at a body temperature of about 40°C (Williams, 1958a; Boone & Huston, 1963). Yet, even here, the testes are sensitive to temperature because exposure to lower temperatures accelerates spermatogenesis and destruction of the germinal epithelium occurs if the testes are subjected to higher temperatures (Williams, 1958a, b).

The ultimate causes of the timing of the mating season in the hyrax are, therefore, probably made up of a combination of several environmental factors and their relative effects on both young and adult animals. Thus, it would seem that selection pressures have acted so as to fix all phases of reproduction temporally in relation to annual changes in the environment, and that they have done so in a way that is most advantageous to the survival of the species.

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The Place of the Seasonal Breeder in Research on Male Reproduction

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Summary: Results are presented from investigations on the rock hyrax and the bat to illustrate how research on seasonally breeding mammals may yield helpful information that is relevant to the problem of male contraception. Attention is focused particularly upon the endocrine control of seasonality and on the response of the epididymis to different hormone levels.

It is reported in the rock hyrax that at the height of the mating season when testosterone levels are at their peak, there is a massive release of LH from the pituitary, suggesting that at these high levels, testosterone does not inhibit gonadotrophin release. Other hormonal changes during the mating season of this animal are discussed.

The apparent asynchrony between the endocrine and exocrine functions of the bat testis indicate a corresponding asynchrony in the release of LH and FSH. Very high concentrations of testosterone have been found in the testis during spermatogenesis, and although levels fall during hibernation, considerable quantities continue to be found throughout the winter.

The dissolution of excess spermatozoa in the epididymis of the hyrax at the end of its mating season also is described and illustrates that under certain circumstances, spermatozoa may be spontaneously removed by the epididymis. Various ultrastructural changes in the epididymis of the hyrax are also described, and their possible significance in relation to epididymal function is discussed.

It is suggested from these examples that male seasonal breeders are useful in contraceptive research by affording a new dimension to the subject that is not available by using conventional laboratory animals.

Problem

Research on the hormonal regulation of sperm production frequently demands removal of various organs of reproduction and hormone replacement as a means of unraveling all the complex processes involved. But an operative procedure such as

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castration is fairly radical, and it would seem that a study of gonadal changes in seasonally breeding male mammals might have advantages in that intermediate stages of stimulation or regression may be studied.

Gonadal changes in the seasonal breeder are temporally quite unique and difficult to mimic experimentally. Stimulation of the testis at the beginning of the mating season is much more rapid than similar stimulation at puberty; yet, at the end of a season, regression of the testes is comparatively rapid. Nevertheless, this biological regression does take some time to be completed and is not, therefore, comparable to castration where hormonal change is much quicker and more dramatic. The seasonal breeder thus allows regressive changes to be studied in more detail since they are progressive and extend over a period.

Most of the data referred to in this paper concern the male rock hyrax (*Procavia capensis*) which is an interesting example of a seasonal breeder [11], but reference will also be made to the hibernating noctule bat (*Nyctalus noctula*) since it displays several special reproductive characteristics. An attempt is made here, therefore, to determine whether or not the mammalian male seasonal breeder has a role in contraceptive research.

Materials and methods

The information presented and discussed in this paper is an attempted synthesis of work produced by a number of people in this laboratory. Most of the work on the rock hyrax has been carried out by Dr. *R. P. Millar,* now in Cape Town; the work on bats is entirely the work of Dr. *P. A. Racey* of Liverpool; and the electron microscopy has been largely undertaken by Miss *F. Suzuki,* at present in Liverpool.

In the rock hyrax, a variety of conventional histological techniques were used for examination of the male tracts and pituitaries, but this aspect of the work will not be discussed here, and data on hormone levels will be primarily used.

The testosterone content of testes in both hyrax and bats was assayed by means of a standard radioimmunoassay. This assay was developed in Liverpool by *P. H. Rowe* (unpublished) and involves the use of an antibody raised to BSA linked to the 3 keto group of testosterone.

Pooled pituitaries (4 in each group) were homogenized in physiological saline and assayed for LH by the ovarian ascorbic acid depletion bioassay [13] and for FSH by the method of *Steelman* and *Pohley* [21].

PMS, HCG, and testosterone were injected into animals while stilbestrol was administered by force-feeding.

Castration of hyrax must be done by flank laparotomy since the testes are situated permanently in the abdomen.

For electron microscopy, small pieces of tissue were fixed in phosphate buffered 2.5 % glutaraldehyde for about 24 hours and then stored in phosphate buffer alone. Material was obtained immediately after death by shooting in the field or under anesthesia in the laboratory. It was not possible to perfuse any of the animals, so all material was fixed by immersion.

It was postfixed in buffered 1 % osmium tetroxide for 3 hours, dehydrated, and embedded in Epon 8.12. Sections were cut on an LKB ultratome and stained with water-saturated uranyl acetate for one hour and with lead citrate for 20 minutes. Sections were then examined using the JEM-7A electron microscope.

Results

From abundant data obtained in this work, only those results that are considered relevant to the subject of this particular paper will be presented.

Hormonal activity at different stages of the mating season

Hyrax: Pituitary histology indicated an increase in gonadotrophin production at the height of the mating season, and testosterone production seems to follow a similar pattern. However, pituitary LH shows a sharp decline before testosterone levels reach their peak in the testis, suggesting that there is a substantial release of LH just before the peak of the season (Fig. 1).

Estimation of pituitary FSH yielded equivocal results, but there was a marked increase in FSH content at the end of the mating season, that is in May.

The administration of gonadotrophin (PMS) to the sexually quiescent male hyrax at the end of the mating season produced renewed enlargement of the testis, and this progressed in response to continued treatment and ceased upon removal of the exogenous hormone. But there was some tenuous evidence that PMS given before the mating season elicits no response by the testis.

The pituitary content of LH during the mating season displayed a biphasic change, declining well in advance of the peak of the season, but rising again at the end of the season. The decline was relatively sudden and indicated a rapid release of the gonadotrophin.

Bat: Results have showed that considerable quantities of androgen are produced during hibernation even when spermatogenesis has ceased.

Epididymal activity at different stages of the mating season

Hyrax: Degenerating spermatozoa were to be found in the epididymis early in the mating season, say about March and again toward the end of the mating season, although actively motile spermatozoa were to be seen in the cauda epididymis

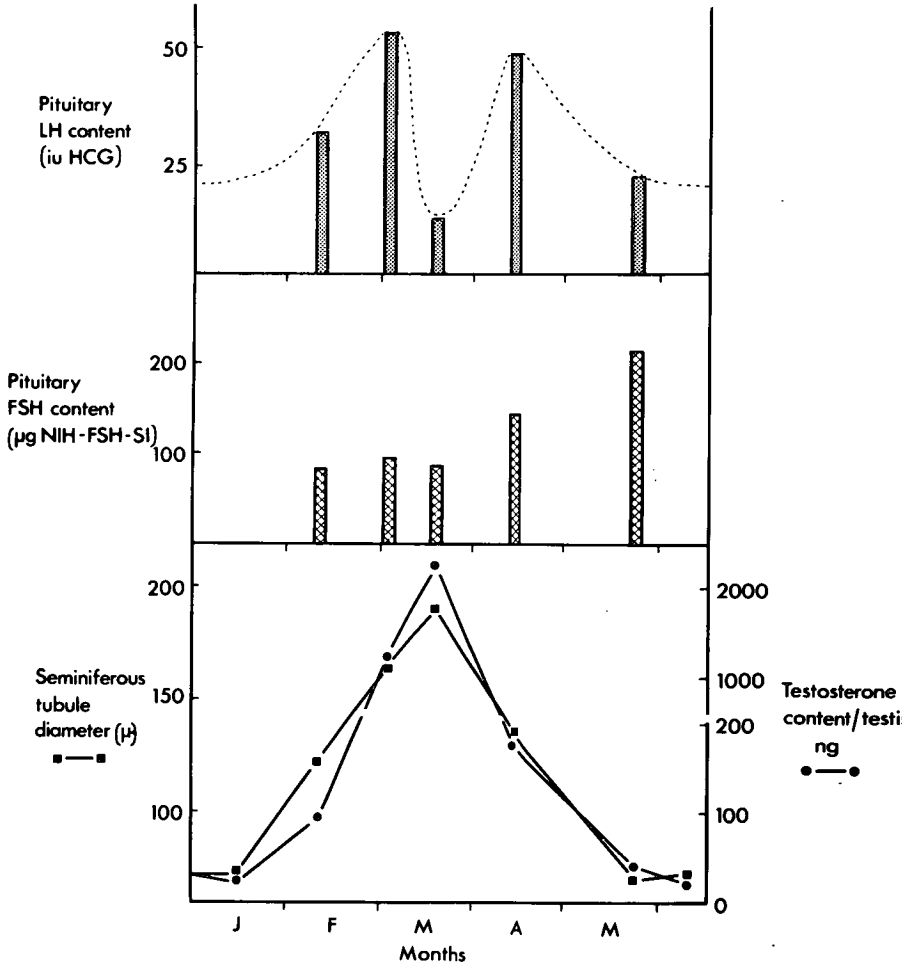

Fig. 1. Seasonal changes in pituitary content of LH and FSH in relation to changes in seminiferous tubule diameter and testis content of testosterone 1 i.u.; HCG approximately equal to 2 μg NIH-LH-SI (from [12])

for some considerable time after the testis had ceased to function. This condition is even more pronounced during the period of hibernation in bats. (Fig. 2).

At the height of the mating season, the principal cells of the hyrax epididymis are fairly typical of those found in other eutherian mammals, in that they have a well-

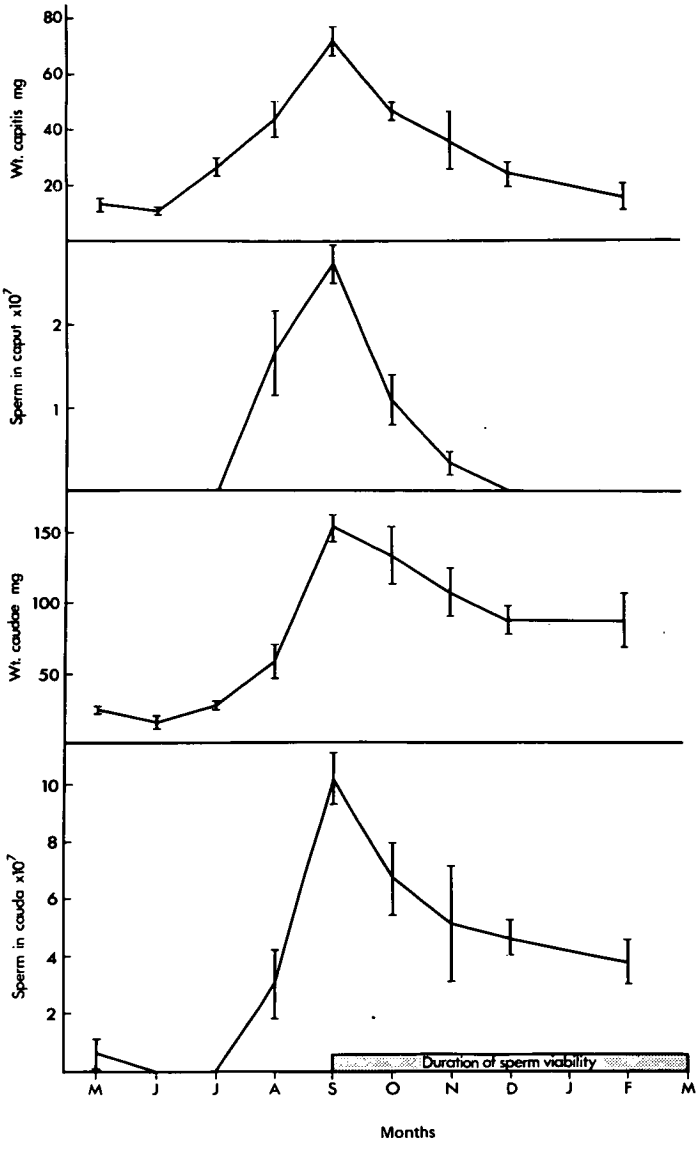

Fig. 2. Seasonal changes in the epididymis of the bat illustrating also the duration of sperm viability (from [16])

developed rough and smooth endoplasmic reticulum, an extensive Golgi apparatus, and several dense bodies. The fundamental structure is similar throughout the length of the duct, although there is some slight regional variation. However, at the beginning of the breeding season, in some specimens, some principal cells are seen to be much darker than normal. These dark cells gradually disappear as the season progresse as animals reach the peak of sexual activity, and as their testes attain their maximum size. The disappearance of these cells, therefore, coincides with the arrival of a significant number of spermatozoa in the epididymis. The cells reappear as the season passes, and, toward the end of the season, they are seen to contain may autophagic vacuoles.

At the height of the mating season, the cells from the cauda epididymis are seen to have a number of lysosomes mainly in the Golgi region, but very large dense granules are also to be seen around the nucleus. These large granules would seem to be residual bodies [4], and it is interesting that their appearance follows the removal from the epididymis of spermatozoa that have arrived there too early in the season and have thereby perished. It certainly looks as if these large bodies result from the ingestion of material (sperm debris?) from the epididymal lumen. Similar large bodie or granules are to be seen in the basal cells of the hyrax epididymis, and ostensibly they persist there for a longer period than in the principal cells.

The fact that these bodies appear after a period of sperm disintegration led us to enquire into the fate of epididymal spermatozoa, unwanted but extant at the end of the breeding season. Examination of epididymal contents under the light microscope has revealed that these spermatozoa undergo head changes that indicate a process of autolysis [10]. The acrosome swells characteristically, and the whole head takes on a rounded appearance although the acrosome is PAS positive and can thus be defined. The structure bears some resemblance to a cast-off spermatid, but each can be distinguished.

Discussion and conclusions

The search for a chemical agent that is suitable for use in the control of human male fertility has, it must be admitted, proved disappointing. Thus, while further testing of drugs should continue, we are really thrown back to the reality of needing more fundamental knowledge about the physiology and biochemistry of male reproduction

This is not to say that much information is not already available, and it is indeed interesting to consider why, with all this knowledge, man is still unable to control his own fertility. Could it be that, on the one hand, there has been some rather indiscriminate testing of drugs, while on the other, biologists have not been motivated by the urgent need for a male contraceptive? This is one of many questions that might be asked in tackling the problem of controlling male fertility, and in this paper there have been many more questions posed than answers given.

In honesty, the question as to whether a study of seasonally breeding animals does or does not have any relevance to the quest for a male contraceptive might be asked. An affirmative answer depends entirely on one's confidence in basic research, and in a belief that it is necessary and urgent to acquire the fullest understanding of the male reproductive system as a whole. If this confidence exists, there would seem a place for the comparative study of male reproductive processes.

Seasonal breeding by males is not only common in the animal kingdom, but is invariably the rule except among the higher vertebrates. Even then, many mammals produce their spermatozoa only sporadically, and the fact that sperm production can be "switched off," that surplus spermatozoa residing in the excurrent ducts can be removed at the end of a breeding season, and that the whole machinery for these events may be reversed without damage, must surely be of interest to those who might wish to "switch off" sperm production in man. In other words, information about seasonal breeding adds another dimension to our knowledge of male reproduction which might not so easily be available from laboratory animals. Also, the manner in which seasonal animals spontaneously become sexually quiescent offers a rather special model for the study of the endocrine control of male reproduction.

The biological origin of seasonality is presumably associated with the protection of gametes. It is much more evident in females than in males, but its occurrence in males results in considerable economy of gamete production. Seasonality is very evident in male fishes, and also in some of the higher vertebrates periods of spermatogenesis may be relatively brief. Thus, in the salamander and in some species of toads, lizards, and snakes, spermatozoa are stored in the testis so that a supply of gametes may be made available after spermatogenesis has ceased. Seasonality in birds has been the subject of an extensive literature, but here, as in mammals, sperm storage is extratesticular and is undertaken by the epididymis. The hormonal control of the epididymis as a sperm store is, therefore, of some significance in addition to the endocrine control of the testis, and for this reason the mammalian seasonal breeder is especially interesting.

Since human physiology has become so well adapted to environmental change, the evolution of seasonality and environmental cues for seasonal behavior are of little interest in relation to the human problem. But endogenous mechanisms involved in sporadic sperm production are of interest in relation to man. Fluctuations in

hormonal activity and responses to such fluctuations are particularly important examples.

Some of these fluctuations observed in the hyrax are especially interesting. For example, the decline of LH before testosterone levels reach their peak indicates that LH is released before the height of the mating season is reached, and that high levels of testosterone that exist at this time do not inhibit LH release.

It is interesting that the pituitary content of LH shows a biphasic change during the mating season because this confirms the idea that testosterone only inhibits the release of gonadotrophins at a particular level or threshold, since at the end of the breeding season testosterone levels are declining. A similar picture has been obtained in the roebuck and the red deer [1, 8, 19]. In the pubertal ram, the pituitary concentration of LH decreases while testicular activity increases [20], and *Davidson* and *Bloch* [3] have fairly recently shown that prepubertal rats are more sensitive than adults to the inhibition of LH release by testosterone. *Wells* and *Zalesky* [23] also stated that in ground squirrels inhibition of the pituitary by the testis varied throughout the year.

One is tempted to speculate from this evidence that there may be a factor which in seasonal breeders is regulated by the environment, which allows the synthesis of LH to begin when the factor is present at a certain level, but which desensitizes inhibitory feedback sites at another level. This could explain the buildup of LH in the pituitary being followed by a relatively sudden and rapid release. On the other hand, the apparent change in the sensitivity of feedback sites to androgen could be endogenous in origin and reflect a change in the type of steroids produced by the testis at the peak of the mating season. If, for example, more androstenedione were produced, it is known that this is less potent than testosterone with respect to its ability to inhibit LH release [2]. Alternatively, the current interest in "inhibin" [18] might have some relevance to a problem of this kind.

Looking more closely at the mechanisms involved, it might be asked also exactly how testosterone synthesis is inhibited by a lack of LH. In this connection, the enzymes concerned in testosterone synthesis are a relevant consideration. If it is these enzymes that are especially susceptible to LH depletion, then one would expect to find an accumulation of testosterone precursors in the testis during periods of sexual quiescence. *Lofts* and *Marshall* [9], for example, showed that accumulated lipid in the seminiferous tubules of birds contained progesterone and its metabolites. A similar situation might prevail in seasonally breeding mammals. However, *Lincoln* (unpublished investigations) does not believe that this holds true at least for the testis of the red deer. Nevertheless, the subject is worthy of closer investigation in the future.

In May, at the end of the mating season of the hyrax, a high content of pituitary FSH indicates that there is no release of this hormone at this time, and effects of

this kind offer an interesting basis of comparison in other species. It is desirable, for example, that a contraceptive in man is selective, and that its action leaves the function of the Leydig cells unimpaired. The reaction of the pituitary to different circumstances is, therefore, important, although a sharp decline in testosterone does not usually affect male sexual behavior for some considerable time. Ideally though, we need to "switch off" FSH and maintain normal levels of LH if we may assume that overlap in function between the two hormones is not particularly significant in this context. Is such asynchrony of the two major gonadotrophins possible? Again there is evidence from seasonally breeding mammals that it is. Generally, even in seasonally breeding mammals, the endocrine and exocrine function of the testis are closely synchronized, but *Racey* [15] has shown that hibernating bats are exceptional and produce significant quantities of androgen during the hibernation period when spermatogenesis has ceased. There are also cases of the reverse situation occurring so that in the Australian brush-tailed possum (*Trichosurus vulpecula*) [5] and in the loris (*Tardigradus Lydekkerianus Cabrera*) [17], Leydig cell activity – as indicated by the state of the accessory glands – fluctuates cyclically in spite of spermatogenesis being maintained throughout the year.

The administration of gonadotrophins to the hyrax indicates that the testis may, under certain circumstances, be refractory to gonadotrophins. This makes an interesting comparison with the common frog where again gonadotrophin will not restore spermatogenesis if given to a sexually quiescent animal [22]. However, stilbestrol may inhibit the development of testicular activity in the hyrax, but, at the peak of the season, the testes do not respond to stilbestrol. Here is further evidence that gonadotrophin release is not adversely affected by steroid when the testis is at the height of its activity.

Epididymal function in seasonal breeders is also of particular interest in that the duct may be called upon to remove excess spermatozoa at the end of a season and yet be prepared for the maturation and storage of new spermatozoa at the beginning of the next season. Since it is well known that epididymal function is androgen dependent, it is to be expected that withdrawal and replacement of androgen in a seasonal breeder will need to be timed carefully. But in the hyrax, motile spermatozoa may be seen in the sperm store (cauda epididymis) even when the testis is quite small [6], and in the noctule bat (*Nyctalus noctula*) spermatozoa may be stored in the cauda epididymis for at least five months during hibernation [16]. It might be added also that this is not due to a temperature or nutritional effect because the situation is no different in noctules that are aroused and fed during the winter. Therefore, it is evident that in both the hyrax and the bat spermatozoa may be stored in the epididymides for a considerable time after the testis has ceased to function. In the noctule bat, testosterone production appears to be at its height in September, and certainly the cauda epididymis is crammed with spermatozoa at this time, but even at the beginning of the calendar year, when testosterone is at

its lowest level, mature spermatozoa remain viable [16]. From this evidence, it appears that the cauda epididymis of the hyrax and the bat may be functionally maintained by very low levels of androgen and are thus relatively insensitive to fluctuations in male sex hormones. In the hyrax, other accessory organs such as the seminal vesicles and prostate, by contrast, are rather slow to respond to injected testosterone [10, 12]. The situation apparently differs from that in laboratory animals in which *Prasad* et al. [14] have claimed that the epididymis is especially sensitive to fluctuations in androgen.

Seasonal changes in the cytology of the epididymis, some of which are reported here, have also proved useful in adding to our general knowledge of epididymal function, although the dark cells reported here are probably artifacts of fixation. Nevertheless, they demand further inquiry.

The onset of the breeding season is not entirely comparable to puberty because in the hyrax the epididymis is not initially prepared to receive spermatozoa, and they do not survive. Thus, early in the season many degenerated spermatozoa are to be seen [10]. By contrast, at the end of the breeding season, the fine structure of the cells in the cauda epididymis remains unchanged in spite of testicular regression, which again points to the relatively low-level requirement of these cells for androgen.

The observation of large dense bodies around the nucleus of epididymal cells immediately after periods of sperm degeneration is suggestive of the fact that they might be associated with the absorption of sperm debris. However, this must remain speculative at the moment. The occurrence of these bodies in basal cells presents a problem. Is it possible that they could move from one cell to another? Or could it be that a principal cell might become transformed into a basal cell? Perhaps the granules in basal cells are specific and are elaborated by the basal cells themselves. These are rather curious events, and investigations on a wider range of species should prove helpful in understanding them.

Only a few examples of information from seasonally breeding mammals have been given, but it is hoped that they will suffice to indicate that male seasonality is relevant to research into the control of male fertility. These animals provide a unique model for studying the endocrine control of testicular function and the maturation and preservation of spermatozoa, as well as possible destruction and removal of spermatozoa by the cells of the epididymis.

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Some new advances in studies of the epididymis.

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THE ROLE OF THE EPIDIDYMAL CELLS IN SPERM SURVIVAL

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INTRODUCTION

Spermatozoa do not remain viable in the epididymis indefinitely and their ability to fertilize is lost after a variable period according to species (1, 2, 3 & 4). But the fate of epididymal spermatozoa, if they are not removed by ejaculation, is not absolutely clear in all species, although in rams, Lino, Braden and Turnbull (5) have produced evidence that unused spermatozoa overflow into the urethra and are expelled in the urine. The question of whether or not some spermatozoa may normally be disposed of within the epididymis is, however, not resolved. Certainly a mechanism exists for eliminating them from the lumen, since they may disappear from the duct in experimental cryptorchidism (6) or when the testes are removed (7 & 8). But this is not to say that the mechanism is invoked in normal animals as a result of prolonged sexual rest. Moreover, from earlier observations, it appears that when the testes are removed, mature spermatozoa in the tail of the epididymis are obliterated much more rapidly and completely than they are in cryptorchidism, where sperm remnants remain in the lumen for some considerable time after the operation. It is possible, therefore, that either more than one mechanism exists in the epididymis for the elimination of spermatozoa or that the efficiency of any one mechanism depends upon the experimental conditions imposed. The present work was undertaken in order to determine whether either or both of these possibilities provide a reasonable explanation for the observed differences in response to castration and artificial cryptorchidism.

An obvious difference between these two experimental conditions lies in the level of circulating androgen, for although there is evidence that androgen is ultimately depressed in artificial cryptorchidism (9), the effect cannot be compared to the drastic reduction in steroid that follows castration. Conditions in a male seasonally breeding mammal may be comparable, however, during periods of sexual quiescence. It was thus considered worthwhile, in the context of the present work, to examine also the epididymis of a seasonally breeding mammal, particularly in the light of Millar's finding (10) that in the rock hyrax (Procavia capensis), spermatozoa disappear from the lumen of the epididymis at the end of the sexual season.

MATERIALS AND METHODS

The problem was approached by subjecting one group of laboratory animals to experimental cryptorchidism and another group to bilateral removal of the testes (gonadectomy). The results obtained were then compared with changes in the epididymis occurring during the sexual season of a seasonally breeding male mammal. Golden hamsters (Mesocricetus auratus) were used as experimental animals and the mole (Talpa europaea) was chosen as the seasonally breeding mammal.

The activity of the epididymis was determined in each animal by examining the fine structure of the lining epithelium in the cauda epididymidis or terminal segment of the epididymis (11) and features of the principal cells and clear cells were selected as being of special interest.

After each animal was killed, both epididymides were removed, together with the testes when present, and they were cleaned of fat in the more proximal regions so that the whole epididymis was clearly visible. Notches were then cut in the duct with ophthalmic scissors to aid penetration of the tissues by the fixative and the whole organ was immersed in 2.5% of phosphate-buffered gluteraldehyde for about 24 hr. A 3 mm^3 piece of tissue was then cut from the terminal segment and washed with phosphate buffer, post-fixed with 1.0% phosphate-buffered osmium tetroxide and dehydrated in the usual series of alcohols. The material was embedded in Epon 812 (TAAB Laboratories) and cut with a diamond knife, using an LKB ultratome, and stained with uranyl acetate and lead citrate before being examined with a JEM 7A electron microscope.

In all the animals, it was found that when the cauda epididymidis was slit with a knife or scissors the luminal content often fountained out into the open, but if it did not, it was clearly visible on the surface of the duct and was easily placed onto a microscope slide for examination. Samples were collected routinely from the epididymis in each animal. The motility of the spermatozoa was observed and subjectively assessed before and after dilution with Krebs ringer phosphate solution and their morphology was examined from smears, after staining with aqueous nigrosin eosin.

Control Material.

Since this work formed part of a wider study of the epididymis plenty of material from normal hamsters was available to serve as

a control, and in any case, details of the fine structure of the hamster epididymis had already been recorded (12).

Experimental Cryptorchidism.

Three hamsters were used in this group. They were subjected to experimental cryptorchidism after being anaesthetized with intraperitoneal Nembutal (Abbott Laboratories). As previously described for the rabbit (13), the testes and epididymides were delivered into the abdomen and secured to the abdominal musculature by means of a ligature through the inverted tunica vaginalis.

Gonadectomy.

This group consisted of eight hamsters, in which the testes were removed under Nembutal anaesthesia and the epididymides were allowed to remain in situ. The operation was carried out through the scrotum, and the testes were removed after ligation of the efferent ductules and the testicular artery.

One of these animals was killed on each of days 2, 4, 7, 14, 21, 28, 35 and 98 days after the operation.

Seasonally Breeding Animals.

Moles were caught live by digging on farms in North Lancashire and were killed by decapitation. Material from the terminal segment of the epididymis was obtained and prepared in the same way as that taken from the hamsters. The animals were collected throughout the year and it was thus possible to examine the epididymides during the sexually quiescent period, at the beginning and peak of the season and during the period of decline in sexual activity.

RESULTS

Control Material.

Our findings on the structure of principal cells in the terminal segment of the golden hamster broadly confirm the description given previously by Nicander and Glover (12). The epithelium is fairly low in this region and the principal cells have regularly distributed microvilli. These cells have quite well-developed organelles and a clearly defined Golgi complex. There is little sign of pinocytosis but multivesicular bodies are present and dense bodies or lipofuscin pigment granules are particularly evident between the nucleus and the Golgi apparatus.

The lumen in this area, which is the main storage area for spermatozoa, is usually packed with spermatozoa that are actively motile *in vitro* and whose cytoplasmic droplets are to be found at the end of their mid-piece.

Experimental Cryptorchidism.

After 14 days there were clear signs of sperm degeneration in the terminal segment of these animals and a marked decrease in sperm numbers. Concurrently, multivesicular bodies appeared to increase in number in the principal cells and there was also a distinct increase in the incidence of dense bodies or lipofuscin pigment granules in these cells (Plate 1, fig. 1.). These bodies persisted in material obtained after eight weeks, although by this time, similar bodies had also appeared in the basal cells. But perhaps the most conspicuous change of all was in the clear cells. At two weeks, about half of these cells were seen to

contain a huge dense body or granule which occupied the greater portion of the cell and displaced the nucleus. Presumably, such extensive granules were formed by the coelescence of smaller ones. At 4 weeks and 8 weeks, this condition prevailed in virtually all the clear cells and is illustrated in Plate 1, fig. 2. There were no obvious changes in the endoplasmic reticulum of the principal cells and the Golgi apparatus also appeared to be unchanged.

Gonadectomy.

Progressive regression of the lining cells of the terminal segment occurred in this group of animals following removal of the testes. The cell organelles became progressively more poorly developed and there were distinctly less multivesicular bodies than usual. Some changes were to be seen even after two days of the testes being removed. Lipofuscin granules again increased in many of the cells, but here they appeared to be associated with autophagi, since they clearly contained cell contents such as mitochondria and residual endoplasmic reticulum. Again, basal cells eventually came to contain granules also, but early on they were less profuse than in the basal cells of cryptorchid animals. Spermatozoa rapidly disappeared from the lumen which became virtually empty within 2 weeks.

Seasonally Breeding Mammals.

In sexually quiescent moles, the principal cells of the terminal segment were obviously inactive. Cell size was greatly reduced, hardly any cell organelles were visible and practically

the whole cell was occupied by the nucleus (Plate 2, fig.1).

At the beginning of the sexual season, there was an enormous increase in the number of ribosomes. This was the most striking feature, although other cell organelles increased also.

At the height of the season, the principal cells achieved a typical size and shape and were obviously in an active state. Compared with the principal cells of this region in the rabbit or mouse, however, the smooth endoplasmic reticulum was poorly developed whilst the rough endoplasmic reticulum was unusually extensive. There were more lipofuscin granules than are normally to be seen in comparable cells of laboratory animals. The general features at this stage of the season are shown in Plate 2, fig. 2.

As the peak of the season passed, several principal cells were seen to regress and large autophagic vacuoles appeared in the cytoplasm. The picture was very similar to that obtained following gonadectomy in the hamster (see Plate 2, fig. 3). Again, the lumen contained no spermatozoa by the time the lining cells had regressed.

DISCUSSION

It is known that when the temperature of the epididymis is elevated as in experimental cryptorchidism or when circulating androgen is removed as in gonadectomy, mature epididymal spermatozoa (see Bedford (14) & Orgebin-Crist (15) for definition) rapidly lose their fertilizing capacity, die and disintegrate. It is now becoming apparent in addition, that the ultimate fate of the products of this disintegration depends upon the functional

status of the epithelium lining the epididymis.

From evidence produced in the present work, it would seem that in experimental cryptorchidism, sperm remnants are taken up by the lining cells of the terminal segment. The large granules seen in an increasing number of clear cells during cryptorchidism indicates a massive increase in absorptive activity. The epididymis has been unequivocally shown to have the capacity for absorption (16, 17), and it has been suggested before that clear cells in the terminal segment may absorb sperm remnants (18). It is unlikely, therefore, that the large granules have an endogenous origin. This applies also to additional lipofuscin pigment granules that develop in the principal cells during cryptorchidism, and extra absorption in these cells is confirmed by an apparent increase in the number of multivesicular bodies. A quantitative estimate of the incidence of multivesicular bodies yet needs to be made, however. It is important to recognise that these changes in the epithelial cells coincide with widespread degeneration in the spermatozoa.

By contrast, the lipofuscin pigment granules, which increase in number in the principal cells of gonadectomized animals, would seem to be autophagic in nature and to be associated with regression of the cells resulting from the deprivation of androgen. Multivesicular bodies, normally seen in the principal cells of the terminal segment, are greatly reduced in number and ultimately, almost all the cell organelles are destroyed. The dense bodies or granules are less homogeneous than those seen in cryptorchid animals, and the fact that they contain remnants

of cell organelles strongly suggests that they play a different role and reflect a different process.

The process of cell regression in the epididymis that accompanies a reduction in circulating androgen may be studied in more detail in a seasonally breeding mammal than in a gonadectomized mammal, because it is much more protracted and thus allows different stages in the sequence of events to be observed. Nevertheless, the ultimate picture is similar in both cases, and in moles, many lipofuscin pigment granules were associated with autophagi.

Suzuki and Glover (19) have suggested that the material absorbed by the principal cells is passed to the basal cells for final dissolution. The basal cells become distended with electron dense bodies, particularly after gonadectomy, and probably act as scavengers of disposable material. This concept will be published in detail elsewhere.

A particular feature of the principal cells of a sexually active mole, is the occurrence of an excess of relatively homogeneous lipofuscin pigment granules distributed mostly below the nucleus. These particular bodies are like those seen in the principal cells of cryptorchid animals and it is concluded that they too indicate some absorption of sperm products. It is conceivable that near the beginning of the sexual season, some of the first spermatozoa to reach the terminal segment fail to survive, because the levels of circulating testosterone are at this time too low to ensure that the lining epithelium is functioning normally.

Testosterone levels at different stages of the sexual season in moles have been measured by one of us (P.A.R.) and will be discussed in detail elsewhere, but spermatozoa can be recovered from the terminal segment of a seasonally breeding mammal before the highest levels of testosterone are reached, so the possibility that they may not survive for very long is feasible. The survival of mature spermatozoa in the terminal segment is dependent upon the maintenance of a special and suitable milieu within the lumen, and this in turn is dependent upon the functional integrity of the androgen sensitive epithelial cells lining the duct (8).

If, in moles, the first spermatozoa to reach the terminal segment do disintegrate, the products of their disintegration might well be seen later in the principal cells, in the form of the more homogeneous type of lipofuscin granules.

This sort of granule is not seen in sexually quiescent moles, however, nor in gonadectomized hamsters, and yet when testosterone is absent, spermatozoa are in some way disposed of within the epididymis, because they may still disappear from the terminal segment of a castrate even when the region is completely isolated by means of ligatures. How then are spermatozoa removed under these conditions when there are no signs of absorption of disintegration products in the lining cells such as are seen in cryptorchidism? Undoubtedly, some spermatozoa will pass into the urethra when the duct is patent, but the answer to the disappearance of spermatozoa <u>in situ</u> probably lies in the observation of Millar (10) that in seasonally breeding mammals sperm dissolution

may be accomplished by lysis within the lumen of the epididymis.

At all events, it is evident that more than one means is available for the disposal of spermatozoa from the tail of the epididymis or terminal segment, in addition to the possibility of their being ejaculated or passively transported into the urethra. None of these means involves phagocytic activity within the lumen, since in none of the conditions described here, were there enough phagocytes present to account for the extensive disappearance of spermatozoa. Nor were whole spermatozoa ever seen to penetrate the epithelial cells. The local removal of spermatozoa does, however, appear to depend on whether or not the lining epithelium is functionally active.

In artificial cryptorchidism, the lining cells of the terminal segment appear to function normally at first. This is borne out by there being initially little change in the chemical composition of the milieu (17), and it is to be emphasized that loss of function and degeneration in the spermatozoa take place fairly swiftly. It would seem then, that in cryptorchidism, the gametes themselves respond adversely to the experimental conditions imposed and disintegration in the spermatozoa somehow gives the signal for the absorption of sperm remnants. It is possible that in vasectomy similar events take place although perhaps at a lower tempo (18). The subject bears closer examination.

It would be surprising if all this absorptive activity occurred in lining cells that are in the process of regression, so when the level of androgen is severely reduced and the spermatozoa die as a result of loss of function by the lining

cells, an alternative means of sperm disposal must be brought in, and lysis of the spermatozoa seems likely to occur under these conditions.

If these explanations are correct, the question arises as to why most spermatozoa are not lysed in normal animals or early in cryptorchidism when androgen levels are high. It indeed seems paradoxical that the most efficient means of sperm disposal is made available when the epithelium is regressed.

Some factor or combination of factors must normally exist to prevent epididymal spermatozoa from being lysed, because it seems unlikely that special lytic agents are elaborated when the epithelium is in a state of regression. It might be that the physical survival of epididymal spermatozoa as well as their functional survival depends upon the nature of the milieu being maintained. Thus, protection from lysis may be achieved from the overall control of epididymal fluid, although it might result from the secretion of some specific substance or group of substances into the lumen.

While being in keeping with our evidence, these suggestions are a matter of speculation, and it is recognised that they only represent a hypothesis upon which further experiments might be based. Chemical and histochemical analyses following gonadectomy and cryptorchidism in laboratory animals (22, 20, 8) have, however, provided a first step towards an understanding of precisely what is happening.

SUMMARY

The effects of experimental cryptorchidism and gonadectomy on the fine structure of the epithelial cells lining the cauda epididymidis (terminal segment of the epididymis) are described in the golden hamster (Mesocricetus auratus) and compared with the changes in fine structure that occur in corresponding cells of a seasonally breeding male mammal during different phases of the sexual season. The mole (Talpa europaea) was the seasonal breeder used in this instance.

In experimental cryptorchidism, mature spermatozoa in the terminal segment disintegrate and become reduced in number, and these changes correspond to the appearance of huge granules in the clear cells and an increase in the incidence of granules in the principal cells lining the duct in this region. The picture is interpreted as indicating that absorption of material from the lumen by the lining epithelial cells has occurred. The possibility of this material being passed to the basal cells is also mentioned.

By contrast, there is no sign of such absorptive activity in the lining cells of gonadectomized animals or in sexually quiescent moles. In these animals, the principal cells have undergone regression and lipofuscin pigment granules are mostly autophagic. Nevertheless, spermatozoa disappear from the lumen in these circumstances and it is suggested that in contrast to experimental cryptorchidism the spermatozoa undergo a process of lysis within the lumen.

From these observations and from evidence in general, it is

suggested that mechanisms exist for the disposal of spermatozoa within the epididymis, and that when spermatozoa disappear in situ, they may either be lysed when they will disappear from view directly or they may disintegrate within the lumen, when the products of their disintegration will be absorbed by the lining epithelial cells.

Thus, it seems that there is a distinction between functional and structural survival in spermatozoa and that the preservation of structural entities even in degenerated spermatozoa, depends upon the lining cells of the epididymis being physiologically active.

ACKNOWLEDGEMENTS

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Plate 1. Fig. 1. 8 weeks after cryptorchidism. Hamster epididymis proximal part of terminal segment. Principal cells of this part keep active structure, and the number of multivesicular bodies (MVB) are many in this field. One of them is associating with large lipofuscin pigment granules (LPG).

MVB = Multivesicular body

G = Golgi apparatus

PN = Principal cell nucleus

x 10,000

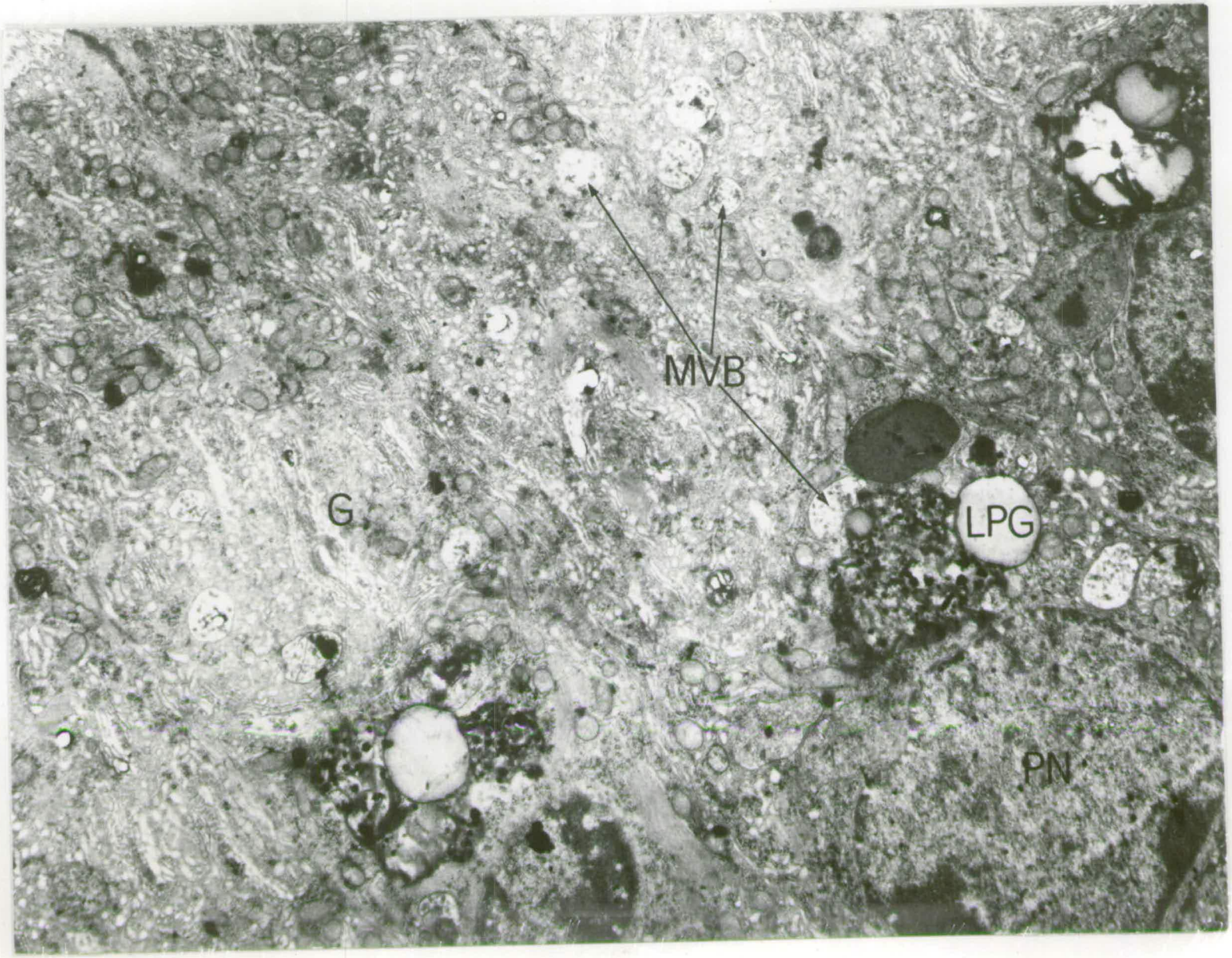

MVB
G
LPG
PN

Plate 1.
Fig. 2. 4 weeks after cryptorchidism. Hamster epididymis, distal part of terminal segment. Picture shows a large granule which occupies almost whole cell volume of the clear cell.

BN = Basal cell nucleus

BL = Basal lamina

x 10,000

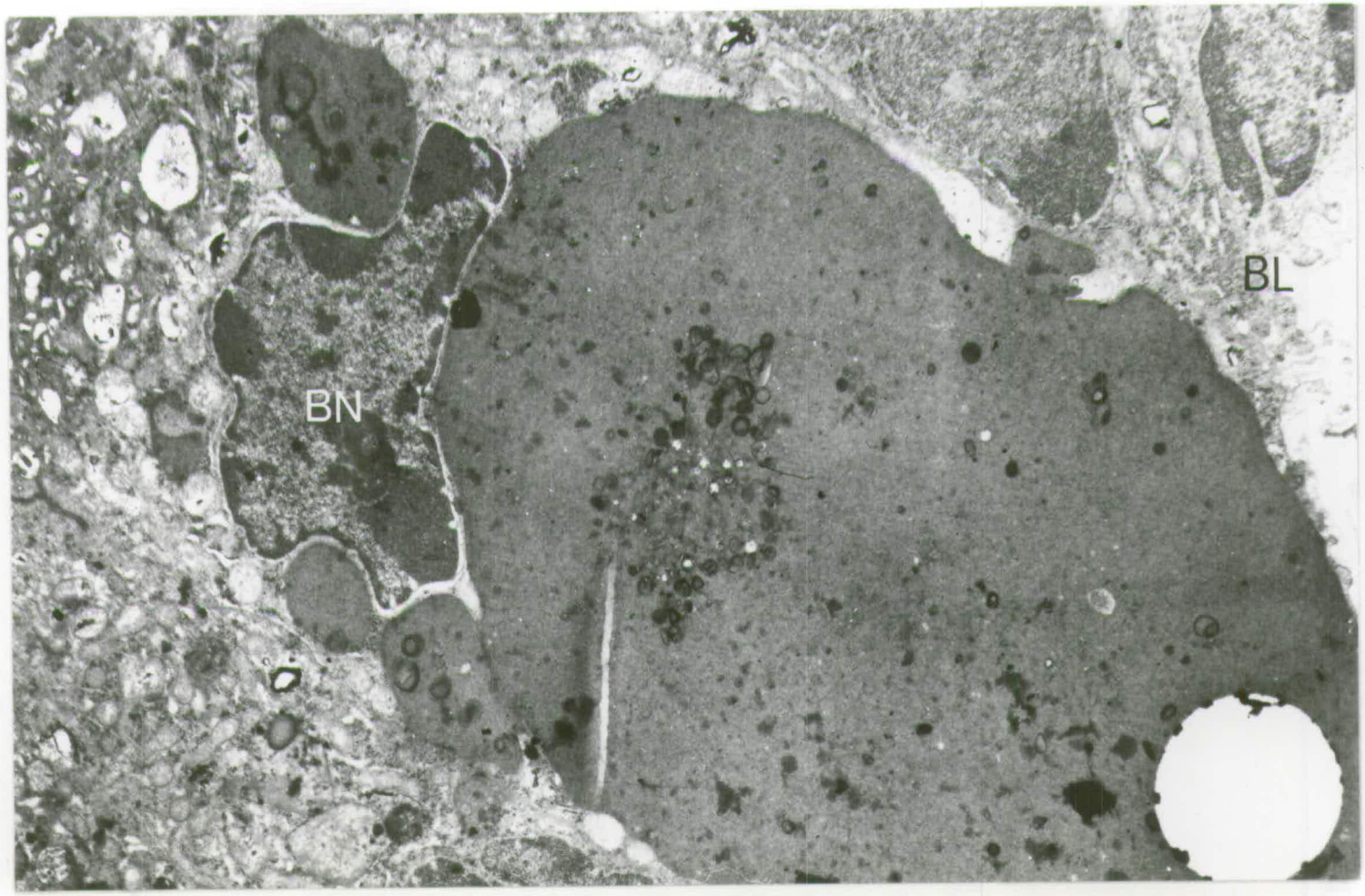

BN
BL

Plate 2. Seasonally quiescent mole (caught September 25th, 1972) Mole corpus epididymidis.

Fig. 1.

LPG = Remnants of lipofuscin pigment granules

PN = Principal cell nucleus

BL = Basal lamina

x 10,000

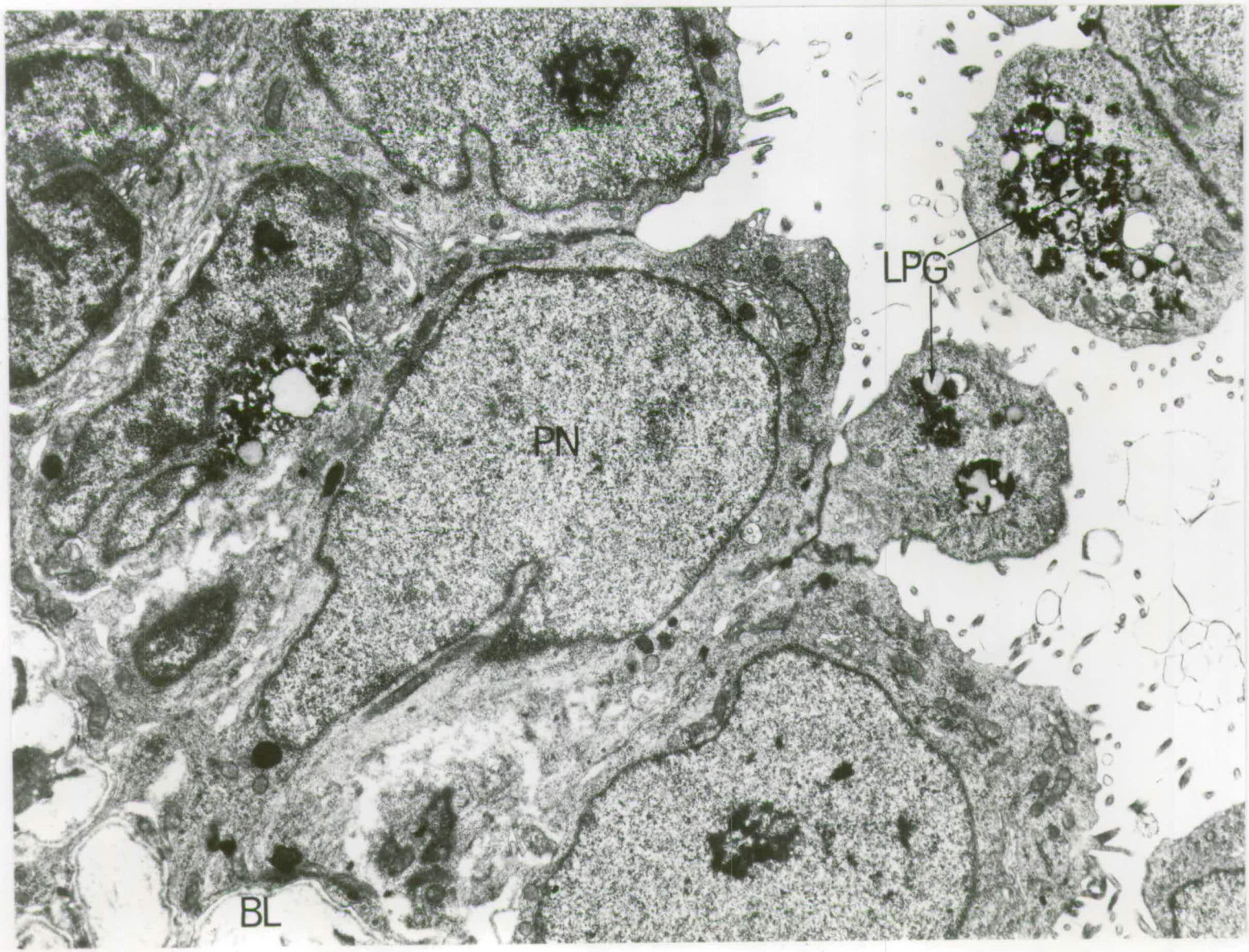

LPG
PN
BL

Plate 2. Seasonally active mole (caught March 11th, 1973)

Fig. 2. Basal half of the epididymal epithelium of body region. Large dense bodies are present most commonly in this region. Rough ER is very well developed in this species, while smooth ER is relatively less well developed in this species.

MVB = Multivesicular body

BL = Basal lamina

PN = Principal cell nucleus

x 10,000

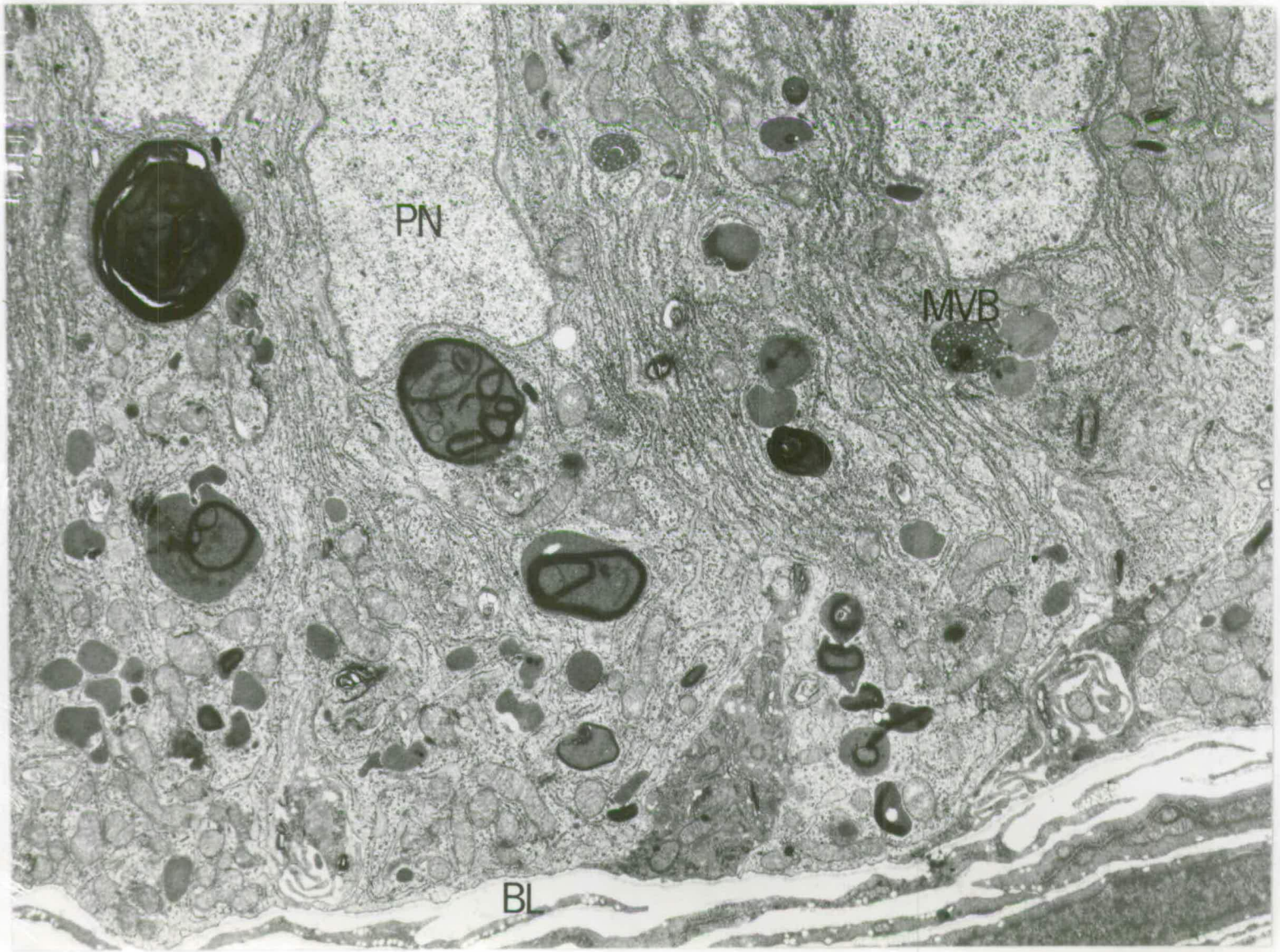

PN
MVB
BL

Plate 2. Seasonally regressing mole (caught May 27th, 1972)

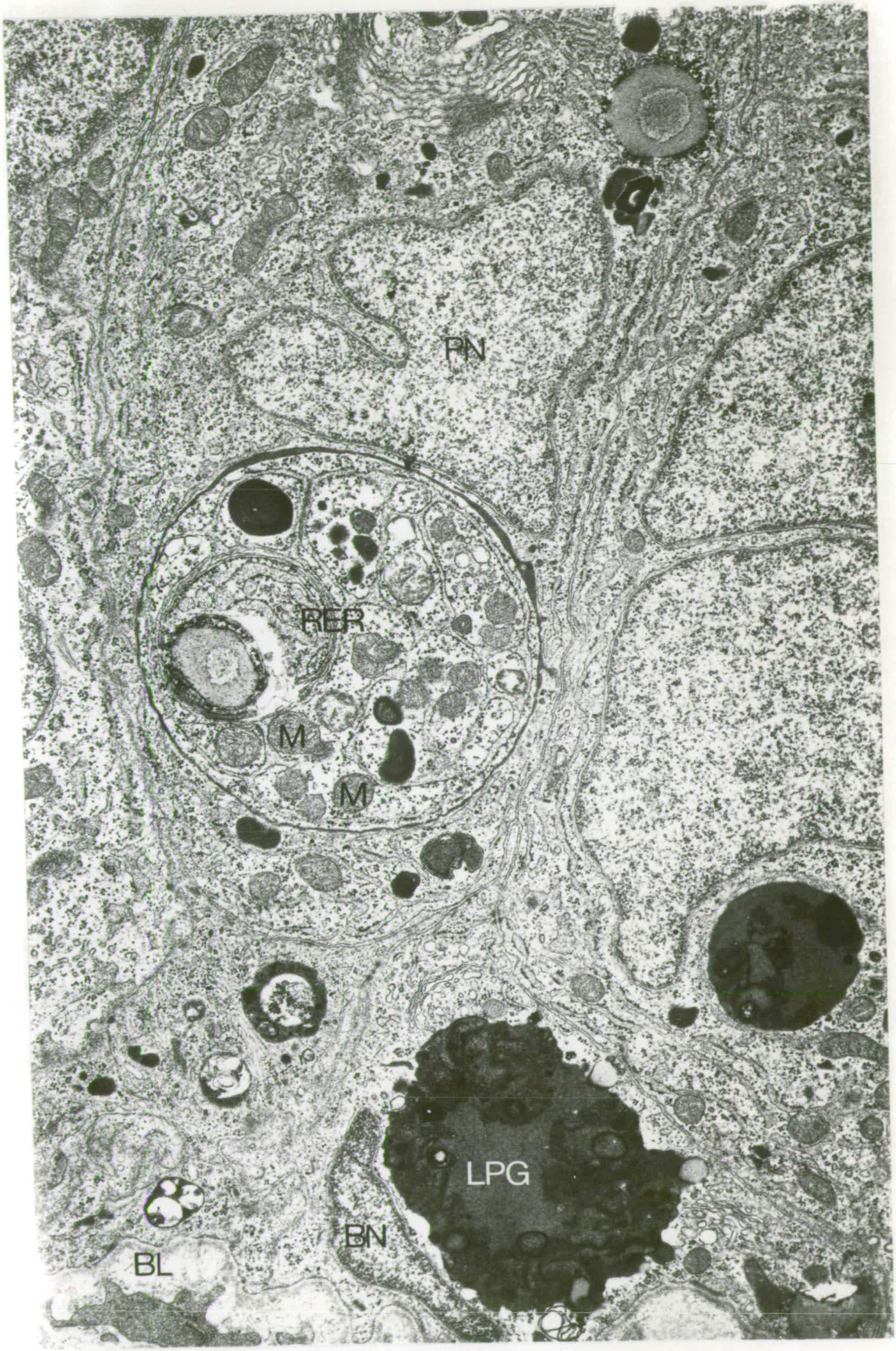
Fig. 3. In the centre of the picture one freshly formed cytosegresome (autophagic vacuole) in the principal cell is presented. Many mitochondria (M) and rough endoplasmic reticulum (rER) are distinguishable in it. Large lipofuscin pigment granules (LPG) are present in the basal wall.

PN = Principal cell nucleus

BN = Basal cell nucleus

BL = Basal lamina

x 20,000


PN
RER
M
M
LPG
BN
BL

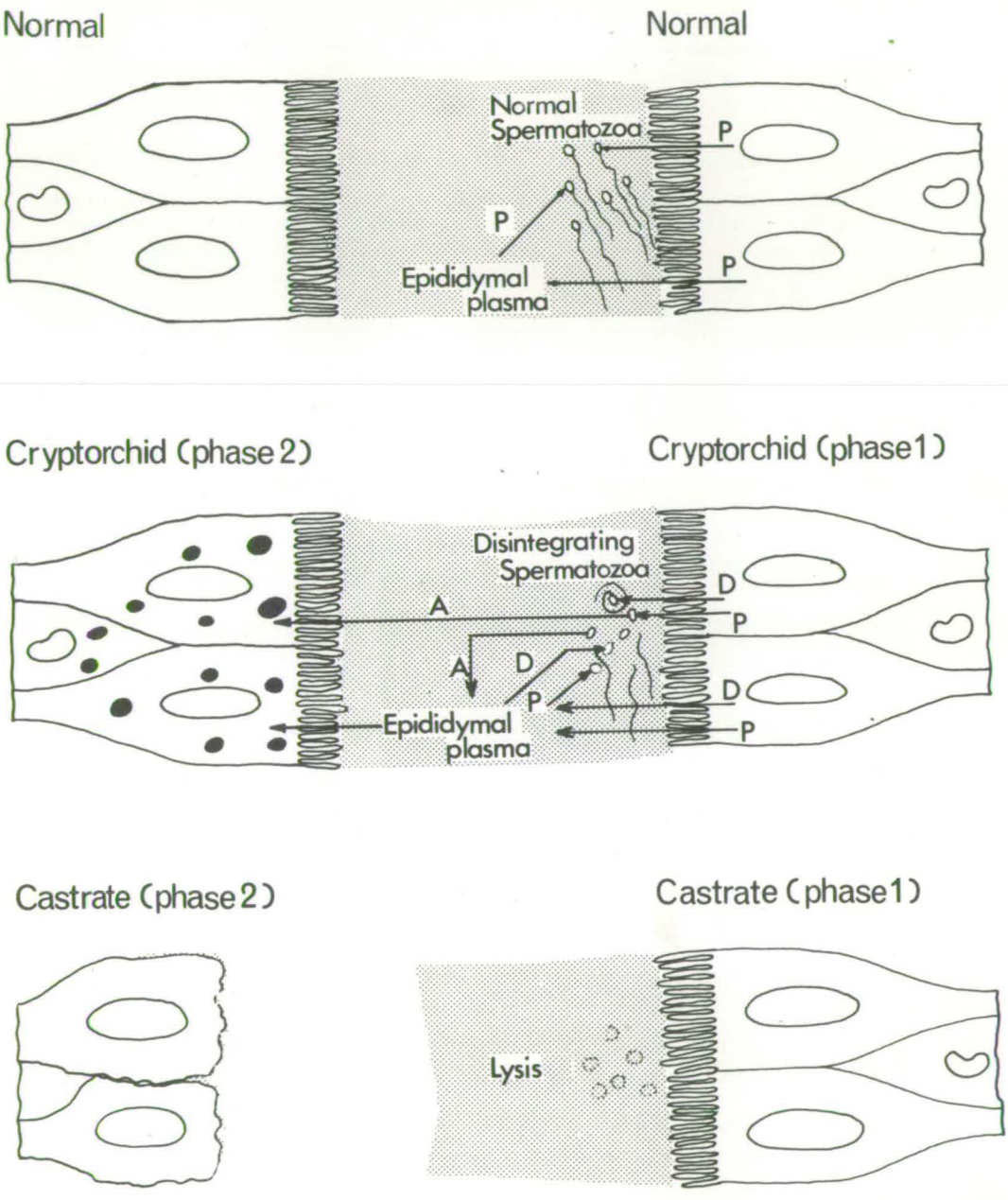
Text
Fig. 1.

Diagrams illustrating theoretical behaviour of epididymal cells and the fate of spermatozoa under different experimental conditions, emphasizing the importance of the epididymal milieu in sperm survival.

P = Protection factor (relating to the basic morphology of the spermatozoa and protecting the cell from lysis.

D = Disintegration factor (bringing about sperm decapitation and degeneration of the mid-piece and tail).

(See text, page 12 in particular)



REACTIONS OF EPIDIDYMAL CELLS

WORLD HEALTH ORGANIZATION

TECHNICAL REPORT SERIES No. 520

REPRODUCTIVE FUNCTION IN THE HUMAN MALE

Report of a WHO Scientific Group

Geneva 1973.

5. EPIDIDYMAL FUNCTION: THE MATURATION AND PRESERVATION OF SPERMATOZOA

The maturation of spermatozoa in the epididymis has been well studied in a wide variety of mammalian species. Thus, most testicular spermatozoa and most of those from the efferent ductules or the proximal segments of the caput epididymis are known to be incapable of fertilization, while those in the distal segments of the duct, namely, the lower part of the corpus epididymidis and the cauda epididymidis, have acquired the capacity to fertilize the ovum if placed in the female tract. The acquisition by epididymal spermatozoa of this capacity to fertilize has not, for obvious practical reasons, been determined in man, but evidence is accumulating from clinical and morphological observations that human spermatozoa do indeed mature in the epididymis. In broad physiological terms, therefore, it is reasonable to predict that the human epididymis will turn out to be similar to that of other eutherian mammals. However, since specific responses to experimental conditions may be expected, this comment must not be taken to mean that results from one species can automatically be applied to another.

The length of the epididymis (about 6 metres in man) is often not fully appreciated; this should be clearly recognized in discussions of frequency of ejaculation, sperm transport, and effects of vasectomy. The tail of the epididymis, where mature spermatozoa are mostly stored before ejaculation, represents a fairly large reservoir, which, because of its distensible nature, can accommodate many spermatozoa. In most mammals, only a small fraction of the sperm reserve in the cauda epididymidis is removed at each ejaculation; it is thus necessary, if the spermatozoa it contains are to be preserved, that prevailing conditions at this level of the duct be conducive to prolonged survival of the spermatozoa in terms of their fertilizing ability.

Two main processes characterize epididymal function—the maturation of spermatozoa and their storage. The rate of passage of the spermatozoa through the duct is also important, particularly from the clinical point of view.

5.1 Maturation of spermatozoa in the epididymis

A number of morphological and physical changes in epididymal spermatozoa accompany the gradual development of their fertilizing capacity and reflect the process of maturation. This process really begins in the testis, and is continued after the spermatozoon is released from the Sertoli cell nad passes into the excurrent duct system. It may be said, therefore, that maturation of spermatozoa is taking place while the cells are still in the seminiferous tubules and the ductuli efferentes, as well as when they are in the epididymis. In fact, in some lower vertebrates, where the testis has a large central canal, the whole process of sperm maturation probably takes place within the testis. In mammals, where such a central canal has become reduced to a relatively small rete testis, much of the process of sperm maturation appears to have come to be extragonadal and is completed in the epididymis. That this duct is derived from the primitive urinary system is of interest, because it suggests that it might be more sophisticated in its function than merely being a conducting tube. This is more than adequately borne out by its histological, histochemical, and (particularly) cytological characteristics.

Nevertheless, the factors concerned in epididymal sperm maturation are not clearly understood. A variety of changes are known to occur in the maturing spermatozoon, however, including the acquisition of the capacity of *in vitro* motility, morphological changes (such as the migration of the cytoplasmic droplet from the neck of the sperm to the end of the midpiece), contraction or condensation of the acrosome in some species, physical changes (such as an increase in specific gravity), a change in light reflecting capacity and in permeability to certain stains, changes in electrophoretic mobility and autoagglutination, and distinct changes in the nucleus itself. The significance of these changes in relation to the fertilizing capacity of the spermatozoon is not clear. Moreover, it has not yet been determined whether sperm maturation is an intrinsic property of the spermatozoa themselves and is thus temporal or whether it depends on the environment within the epididymal lumen. But there is evidence that both factors are involved.

5.1.1 *Studies on spermatozoa*

It is evident that during their maturation spermatozoa undergo a change in their surface nature as well as in their chemical nature, and this phenomenon requires closer scrutiny.

5.1.2 *Studies on the environment within the lumen of the epididymis*

As yet, information on the chemistry of epididymal fluid at different levels of the duct is fragmentary. Workers have tended to concentrate

particularly on one or two components, and in species other than domestic animals it is extremely difficult to obtain epididymal fluid uncontaminated with cellular material. Better methods of cannulation of the duct are called for, so that more comprehensive analysis may be undertaken on a wider variety of species, including man wherever possible.

Axiomatically, consideration of factors controlling the epididymal milieu leads to an investigation of the physiology of the cells lining the epididymis, since they are largely concerned in the elaboration of the luminal plasma.

Although the enzyme 3-α-hydroxysteroid dehydrogenase has been located in epididymal spermatozoa and it is known that testicular fluid has quite a high content of testosterone, there is, at present, little reason to suppose that androgens have a direct effect on epididymal spermatozoa. Available evidence suggests that the influence of steroids on the cells lining the epididymis is much more important.

Another issue that has been neglected is the influence of the contents of the lumen on the activity of the lining cells. It is conceivable that the number or concentration of spermatozoa, or their rate of passage, might influence the function of the lining cells. Certainly, an abundance of degenerated spermatozoa or an accumulation of fluid at any level of the duct modifies the morphology of epididymal cells and possibly their activity.

Research on epididymal physiology should also include a more careful study of blood flow and vascular patterns in the epididymis.

The length of time required for a spermatozoon to mature is still largely unknown, and it would be helpful if this were established or if indeed the process could be achieved *in vitro*. The maturation time *in vivo*, however, is intimately bound with the time and mode of sperm transport in the epididymis.

5.1.3 *Studies on the transport of spermatozoa through the epididymis*

Since spermatozoa are immotile or only poorly motile in the epididymis, the control of peristaltic-like movements of the duct transporting the spermatozoa to the cauda epididymidis is an important subject. Details of the autonomic nervous control of the mechanism should be helpful, and further work should be undertaken on the influence of ejaculation frequency, which is the subject of differing opinions. Knowledge in this field is important for a true understanding of the maturation of spermatozoa and for progress in fertility control.

5.2 Storage of spermatozoa

The fertilizing capacity of spermatozoa is well preserved in the cauda epididymidis, and we need to know how this is achieved. The withdrawal of androgen rapidly destroys the functional ability of spermatozoa, pre-

sumably because of profound changes in the chemical composition of the fluid. On the other hand, the viability of spermatozoa can be impaired by factors such as heat, even when the composition of the milieu does not appear to be significantly changed. More precise information on oxygen tension in the lumen and on the osmotic pressure of the contained fluid is also required in order to understand fully the problem of sperm preservation in the tract.

Spermatozoa do not survive indefinitely in the male tract, however, and after removal of the testes they rapidly disappear even from regions of the tract that are completely isolated by ligation. The mechanisms involved are unknown, and it is by no means clear whether or not they are evoked in intact individuals during long periods of sexual inactivity or following vasectomy. The response of different species in this regard needs testing, with particular emphasis on the human being, but it might be stressed that in animals there is no evidence of widespread classical phagocytosis of spermatozoa in the epididymis.

T. D. Glover.

Rapporteur.

DISCUSSION AND CONCLUSIONS

The thesis opens with some collaborative work that showed for the first time the nature of the sympathetic control of seminal emission. The work was carried out on rabbits and demonstrated the relative role of the lateral areas of the hypothalamus and the adrenal medulla on this control and the contribution of three distinct physiological entities, namely, erection of the penis, seminal emission and ejaculation to the overall process of seminal discharge. It provided evidence to indicate how extreme sexual excitement may result in an especially good semen sample being produced, due to more extensive evacuation of the distal limb of the cauda epididymidis.

About this time, the author was also undertaking his experiments on artificial cryptorchidism in rabbits. These experiments showed that if the epididymis was secured in the abdomen, most of the contained spermatozoa died and degenerated within a matter of two weeks. The first signs of degeneration in the spermatozoa were to be seen after four or five days, when ejaculates showed an increased incidence of spermatozoa with coiled tails. This was soon followed by a characteristic decapitation effect. Since the same pattern of seminal change occurs when the scrotum is insulated, it is evident that the effect is due to a local increase in the temperature of the epididymis. Further experiments showed that there is a difference between epididymal spermatozoa at different levels of the duct in their susceptibility to increased temperature.

Immature spermatozoa in the caput epididymidis appear to be more susceptible than mature spermatozoa residing in the cauda epididymidis. The delay in the onset of seminal degeneration following artificial cryptorchidism or insulation of the scrotum must, therefore, be due to a cumulative effect of temperature on mature spermatozoa.

Another piece of work showed, however, that even mature spermatozoa lose their capacity to fertilize in only two or three days following artificial cryptorchidism. The only morphological indication of this functional deficiency was an apparent retention of the cytoplasmic droplet by many of the ejaculated spermatozoa. It is the opinion of the author that these cytoplasmic droplets may have more functional significance than has hitherto been attributed to them and it is of interest that more work demonstrated a highly specific localization of alkaline phosphatase in the droplets. This enzyme is associated with the transport of sugars across membranes and the very specific behaviour of the droplets during the maturation of spermatozoa casts additional doubt on their presence being purely fortuitous.

The work has also shown that spermatozoa in the cauda epididymidis do not retain their viability indefinitely. This was already known, but the precise effect of sperm ageing on the fertilization of eggs had not previously been demonstrated. Also, this earlier work illustrated conclusively, that the maturation of spermatozoa in the rabbit is not entirely a temporal phenomenon as had been argued before, but that the

environment within the lumen of the epididymis is vital to the process.

Attention was next paid, therefore, to mammals whose testes are normally retained in the abdomen ("mammalian testiconda"), since their epididymides must also be abdominal. The question arose as to whether there were any specific features of these mammals that allowed epididymal spermatozoa to maintain their viability in spite of the duct being located in the abdomen.

The particular subject of this study was the rock hyrax (Procavia capensis and Heterohyrax) but other testicond mammals including the elephant and several African shrews were also examined. The genital organs of the spiny anteater (Echnida), the aardvark (Orycteropus), the armadillo (Dasypus), the sloth (Choloepus), species of anteaters (Myrmecophagidae) and the golden mole (Chrysochloris) have also been studied, but results on these species have not yet been published. However, they confirm in large measure the findings published on the rock hyrax. These findings were that the tail of the epididymis in a true testicond mammal, or at least the homologous part of the mesonephric duct, is situated in the pelvic cavity, and is thus nearer to the surface of the body than it would be if it were adjacent to the testis as it is in a scrotal mammal.

Thus, the work showed that previous terminology concerning the genital ducts of mammalian testiconda was incorrect, and that the ductus deferens, far from being long was, in fact, very short. It demonstrated, moreover, that there appears to

be a tendency in mammals generally for the sperm store to be situated close to the body surface, presumably, because it is cooler there. Such a tendency is also apparent in birds, and in some avian species, the tail of the epididymis lies outside the abdomen in a scrotal pouch, while the testis is situated deep in the abdominal cavity.

These results have enabled the author to put forward a new concept of the evolutionary development of the epididymis in mammals and in collaboration with Professor Nicander of Stockholm, to suggest new regional terms for describing the epididymis, based on the functional evolution of the organ.

True testicond mammals have been shown to have a straight testicular artery and no pampiniform plexus. In addition, the males of many of the species exhibit seasonal sexual activity. These two features suggest that there is much more versatility in the circulation of blood through the testes of these mammals than through the testes of scrotal mammals and that the hormonal control of the gonads is also rather different.

Experiments already completed had shown that when the temperature of the scrotum in rats is increased, blood flow in the testis changes but little compared with that in the epididymis, where it is markedly increased. It could be, that when the temperature of the scrotal testis is increased, it suffers from anoxia or a deficiency of some other circulating substance due to restricted blood flow, but this would not

seem to apply to tne epididymis. The restricted testicular blood flow in conditions of elevated temperature is doubtless associated with the unique vascular architecture of scrotal testis, a special feature which does not characterise the gonads of testicond mammals. Thus, in a sexually active hyrax, for instance, the blood vessels of the testes are seen to be greatly distended. Furthermore, the testes themselves increase in size during sexual activity so that they almost fill the abdomen in much the same way as the testes of some sexually active birds, and in marked contrast to the sexually quiescent organ, which, in the hyrax, is only 2-3 cms in length.

Regular fluctuation in hormonal activity in the rock hyrax is thus quite obvious, and this led the present author to take an interest in the problem of the hormonal control of male reproductive function, particularly in relation to seasonally breeding mammals.

As a result, it was decided that reproductive activity in a male seasonal breeder should be studied more closely and the rock hyrax was again chosen as the experimental animal, since it is a successful breeder and compared with some other exotic species, is readily obtainable. So a study in depth of the breeding of this small mammal was made in collaboration with the author's research assistant, R. P. Miller, who was sent out to South Africa to build up a colony of rock hyrax over a period of two years.

It was shown that at the end of the breeding season in this

mammal, spermatozoa degenerate and disappear from within the epididymis. Such disappearance had previously been shown by the author in the ligated epididymis of artificially cryptorchid rabbits, but the process of dissolution could not be demonstrated under physiological conditions. It was later shown that under these conditions, the process of sperm disappearance was special, in that it resulted from intraluminal autolysis, in contrast to the pathological state where sperm debris appeared to be absorbed by the lining cells.

These observations were combined with estimations of circulating androgen and it soon became clear that epididymal spermatozoa rapidly lose their functional and structural integrity if the level of circulating testosterone is allowed to fall too low.

The dependence of epididymal spermatozoa upon androgen was confirmed by experiments in the golden hamster (Mesocricetus auratus) using fertility trials as the experimental test. Increasingly, it seemed probable that if epididymal spermatozoa were to maintain their viability in the lumen for a reasonable period, the milieu in which they were suspended must also be maintained in an appropriate state. The maintenance of such a state was likely to be dependent upon normal function in the epididymal cells. If these cells were sensitive to androgen, and it might be expected that they would be, then loss of fertilizing capacity resulting from a decrease in androgen would be due to failure of the epididymal cells to elaborate

an appropriate milieu for spermatozoa within the lumen. Alternatively, of course, androgen might act directly on spermatozoa in the duct.

It was, therefore, of interest to explore the effects of castration and androgen replacement on the epididymal milieu and, from further investigations, it became apparent that the survival of spermatozoa in the cauda epididymidis was dependent upon the nature of the epididymal plasma. The effects of withdrawal of androgen on the chemical composition of epididymal plasma in the rabbit have been described and the implications of these changes in relation to sperm viability have been discussed.

Other investigations presented include a histochemical study of the comparative distribution of lysosomal enzymes in mammalian epididymides and electron microscopical studies of the organ. Two review articles attempting to collate the information obtained are also presented.

ABSTRACT OF THESIS

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Title of Thesis CONTRIBUTIONS TO THE BIOLOGY OF REPRODUCTION IN THE MALE ANIMAL WITH PARTICULAR REFERENCE TO THE STRUCTURE AND FUNCTION OF THE EPIDIDYMIS.

This thesis is mainly concerned with a biological study of the mammalian epididymis and embraces a series of investigations that were begun in 1956.

The introduction to the thesis consists of a broad review of the subject of male reproduction. This review is at present in press for publication in the Physiology Section Series 1 - Reproductive Physiology. Ed. R. O. Greep, Medical and Technical Publishing Co., Lancaster. Chapter 9, pp. 1-57.

Then three early papers are enclosed as a prelude to the thesis and these are intended to provide background information only. The main subject of the thesis is opened with a paper on the innervation of the male reproductive tract in rabbits and in this paper the physiology of seminal emission is investigated. The work continues with an investigation of the effects of artificial cryptorchidism on epididymal spermatozoa and includes a review on the whole subject of temperature and male reproductive function. The effects of variations in scrotal temperature on blood flow in the testis and epididymis of the rat have been examined and the possible physiological significance of these effects is raised.

Factors concerned with the maintenance of viability in epididymal spermatozoa have been studied and the effects on fertilizing capacity of ageing in epididymal spermatozoa, of castration and of the administration of exogenous hormones, are also described.

The significance of the luminal environment within the epididymis in relation to sperm maturation and sperm survival has been illustrated and the influence of circulating androgen on the chemical composition of epididymal fluid has been demonstrated. The author has speculated about the importance of the epididymal milieu in the maintenance of fertilizing capacity in the spermatozoa and in relation to their fate in the epididymis.

A comparative histochemical study of mammalian epididymides is presented, illustrating the distribution of lysosomal enzymes. The possible significance of some of these epididymal enzymes in relation to sperm maturation and survival is discussed.

The thesis mostly consists of an account of experimental work carried out in the laboratory, but a major aspect of the work concerns a study of the epididymis in African testicond mammals (mammals with abdominally situated testes). These investigations have led to a new concept of the evolution of the mammalian epididymis and they represent a new approach to the biology of the organ.

Since many African mammals are seasonal breeders, the above studies have also stimulated the author's interest in the hormonal control of

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male reproduction in general and in the control of the testis and epididymis of seasonally breeding male mammals in particular. Most attention has been paid to the rock hyrax (Procavia capensis), but results on other species are also mentioned, especially in a paper presented to the Schering Workshop on male contraception. In this paper, the relationship of comparative studies of the epididymis to the problem of male contraception in the human being is explained.

In a later paper, the subject of the dissolution of spermatozoa in the epididymis is dealt with, and some electron microscopical findings associated with the disintegration of epididymal spermatozoa are presented.