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Control Mechanisms of Testicular Differentiation [and Discussion]

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Control mechanisms of testicular differentiation

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[Plates 1–4]

In this paper the importance of unknown factors responsible for the initial differentiation of a gonadal primordium is stressed. The hypothesis that in the absence of testis determining genes (TDG) the indifferent gonad is programmed to become an ovary is considered further. The TDG(s) are expressed only among cells already marked as gonadal cells, and they seem mainly to change the chronological sequence and intensity of expression of processes common to both sexes. The chronology of the normal events necessary for testicular differentiation and the fact that some of these events can be dissociated from one another under experimental conditions *in vitro*, suggest that many genes are involved in testicular differentiation and that the so-called testis-determining genes are probably regulatory genes.

Introduction

The embryonic development of organs like the lung or the pancreas, although intensively studied, is still incompletely understood. These organs originate from a bulging of a definite part of the primitive endodermal gut epithelium into the underlying mesodermal mesenchyme. Epithelial–mesenchymal interactions eventually end in the embryonic 'determination' of a pancreatic or a lung primordium whose fate is fixed for life. Characteristic proteins such as insulin, glucagon or lipase are synthesized.

The cellular influences that initiate the development of a definite organ in a definite region of the body are still unknown. It would seem that the cellular environment turns on some gene(s) in a definite area, and that 'organ-determining genes' come into play. These genes rapidly govern the morphological and physiological specialization of a localized part of the embryo.

The origin of the gonads, on the inner side of the mesonephroi, involves mesodermal cells, namely the coelomic epithelium and the underlying mesenchyme, and germ cells that have migrated into the region. The first recognizable gonadal primordium is the so-called undifferentiated (indifferent) gonad whose characteristics require some consideration.

The indifferent gonadal primordium and TDG(s) expression

Some 'organ-determining gene(s)' probably controls the initial formation of the gonadal primordium in a way similar to that conjectured for other organs. But unlike the lung or the pancreas, the fate of this primordium is not yet determined. It is a transitory structure than can become either an ovary or a testis or a hermaphroditic organ, or it can abort.

(*a*) *Sexual significance of the 'indifferent gonad'*

In the past, it had long been accepted that the indifferent gonad was hermaphroditic, i.e. composed of a male and of a female component, and that the final sex of the gonad resulted from the competition of two discrete and sexually opposite types of cell, male and female, under genetic control (Witschi 1951, 1957).

Several years ago, one of us (A.J.) introduced the concept that the unknown locally expressed 'gonad-determining gene(s)' actually initiated an 'ovary-determining' programme (Jost 1965, 1970). In males, this programme is upset when, at a definite time and for still unknown reasons, the testis-determining genes (TDGs) become activated. Morphogenesis and cytodifferentiation are accelerated according to the male pattern, for instance by calling into action genes that would have remained temporarily inactive in a developing ovary. Thus Voutilainen & Miller (1986) recently observed an early and time-dependent amount of mRNA for cholesterol side-chain cleavage enzyme and for 17 β-hydroxylase/17,20 lyase in the human foetal testis, whereas these mRNAs remained scarce in the ovary.

This concept is dramatically supported by the so-called 'undifferentiated sex races' of grass frogs, in which all individuals first develop gonads similar to ovaries until the time of metamorphosis. After that time the gonads of 50% of the individuals, the males, undergo a delayed masculinization, involving the disappearance of the large oocyte-like germ cells (Witschi 1930, 1942). It appears that the testis-differentiating gene(s) become active only at metamorphosis, permitting male gonads to differentiate along the ovarian line up to that time. Interestingly, the expression of the TDG seems to be controlled by environmental factors because masculinization is not delayed in the frogs living in mountains or in northern countries (Witschi 1930, 1942).

Another well-known example of delayed ovarian masculinization is afforded by ovaries of freemartins in cattle, which pass through three phases: (1) they first remain similar to presumptive ovaries at the time when testes differentiate in males; (2) next they become stunted for 6 weeks or more; (3) seminiferous cords and Leydig cells differentiate after the third month of foetal age (Jost *et al.* 1972, 1973). In that case testicular organogenesis in presumptive ovaries does not result from the presence of a Y chromosome (because no Y chromosome is present except in blood cells), but from the effect of humoral factors transmitted by blood exchange with the male twin.

(*b*) *Similarities and dissimilarities in testicular and ovarian development*

The developmental prospects for the gonadal primordium are in some measure similar in both sexes. Schematically, the gonads are organs that permit and control the differentiation of the germ cells, and on the other hand are endocrine glands that produce sex hormones. Seminiferous tubules in testes and ovarian follicles in ovaries are formed of homologous cells which both produce the Müllerian inhibitor (AMH or MIS) at one time or another (Vigier *et al.* 1984; Donahoe *et al.* 1987), and which control meiosis. In males, the aggregation of Sertoli cells around the germ cells is a very early event (Jost 1972; Magre & Jost 1980), and meiosis is prevented for a prolonged period of time. In females the follicle cells surround the oocytes only after the germ cells have entered meiosis, and meiotic prophase is arrested in the follicles. In both sexes the germ cells become isolated from direct contact with blood capillaries: they depend strictly on the surrounding cells, which govern the male or female gametogenesis in an unknown way.

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On the other hand, in both sexes the sex hormones, testosterone and oestradiol, derive for the most part from cholesterol; the biosynthetic pathway is cut short in males, and androgens are released, whereas in the ovary androgens serve as precursors in a longer series of transformations.

To a large extent the tools used to make an ovary or a testis are similar, but the TDGs seem to change the chronological order completely, hastening, repressing or reinforcing the expression of genetically controlled processes, which in one way or another also occur in the developing ovary.

It is obvious that a concept expressed so bluntly is too schematical, and that it can be proposed only for the initial stages of gonadal development. Testes and ovaries progressively acquire their own characteristics and properties; other specific genes become activated after the initial sexual orientation.

(*c*) *No TDG expression outside the gonadal primordium*

The testis-determining gene(s) becomes activated only in the gonadal primordium. When germ cells, during their migration period, enter the adrenals rather than the gonadal primordium, they become oocytes and enter the meiotic prophase even in males (Upadhyay & Zamboni 1982; Zamboni & Upadhyay 1983). During the same time and in the same animal those germ cells that entered the testis become non-meiotic spermatogonia. In the adrenal gland neither follicular nor Sertoli cells differentiate, although adrenals and gonads are related structures developed from rather similar neighbouring tissues. The organ patterning, also reflected by the nature of the major steroid produced, is different in the two organs. TDG expression does not belong to the adrenal programme.

Sequences in the normal differentiation of a testis

One obvious way to contribute to the elucidation of the control mechanisms of testicular differentiation consists in carefully scrutinizing how a testis normally develops. An important part of this approach is the recognition of the chronological succession of the processes involved, because testicular differentiation is a stepwise process.

It has long been known (even if it is sometimes overlooked) that the foetal seminiferous cords (the core part of the future seminiferous tubules) differentiate much earlier than do the Leydig cells. In humans more than one week separates the two processes (Pelliniemi & Niemi 1969). As early as 1923, Kitahara drew attention to this point and suggested that the Leydig cells differentiate under the influence of the seminiferous cords. The idea that Sertoli cells might exert this influence has long appealed to the senior author of this paper.

The initial steps of testicular differentiation have rarely been scrutinized with enough precision. We tried to fill this gap by studying rat foetuses. Their age was reckoned from the assumed time of fertilization of the oocyte, which usually takes place at 01h00 or 02h00. Their sex was determined by the sex chromatin test in the cells of the amniotic membrane (Jost 1972).

Figure 1, plate 1, shows the appearance of the indifferent rat gonad. The first event we could recognize in rat foetuses aged 13 days 9 h was the emergence in the course of a few hours of a new cell type in the undifferentiated gonad: large clear cells, which aggregate and encompass the germ cells into the forming seminiferous cords (Jost 1972; Jost *et al.* 1973) (figure 2*a*, *b*, plate 2). Characteristic interdigitations between these cells are seen with the electron

microscope (Magre & Jost 1980). This takes place first in the anterior part of the gonad and extends to the entire gonad during the next 24 h. During the same time a basal membrane progressively forms around these cords (Magre & Jost 1980) (figure 2*c*, plate 2.) By 15 days 13 h the Leydig cells have begun to differentiate (figure 3, plate 3). The myoid cells and the wall of the seminiferous tubules do not become conspicuous until after birth (22 days of pregnancy).

In contrast with the early organogenesis of the testis, ovarian follicles form postnatally. It is also noteworthy that the testicular seminiferous tubules, once established, become permanent even in case of disappearance of the germ cells, whereas persistence of ovarian follicles depends strictly on the presence of germ cells.

It is clear that the differentiation of a testis results from a cascade of processes and probably from the successive activation of many genes.

Interfering with testicular organogenesis *in vitro*

Morphological and endocrine testicular differentiation from undifferentiated primordia of rat foetuses can be obtained *in vitro*. Primordia taken from fetuses aged 12 days 16 h (before gonadal sex can be histologically recognized) or 13 days 9 h (at incipient differentiation), and explanted in a synthetic medium with the mesonephroi, complete their morphological and endocrine differentiation within 3 days. Though growth of these gonads is very limited *in vitro*, some seminiferous cords are formed (figure 4*a*, plate 4) and Leydig cells subsequently differentiate. Production of the Müllerian inhibitor was tested by the *in vitro* test devised by Picon (1969); testosterone and androstenedione were measured in the medium with a radioimmunoassay.

In the first series of experiments (Agelopoulou *et al.* 1984), it was observed that the addition of foetal calf serum, or other sera (Chartrain *et al.* 1984), to the synthetic medium prevented the differentiation of the seminiferous cords (figure 4*b*, plate 4). However, large clear cells, looking like foetal Sertoli cells, were scattered throughout the gonads; these were assumed to be true Sertoli cells because the cordless gonads produced the Müllerian inhibitor, a product of the Sertoli cells (Magre & Jost 1984). Moreover, these gonads contained cells showing 3β hydroxysteroid dehydrogenase (3β HSDH) activity and produced testosterone (Patsavoudi *et al.* 1985). These experiments suggested that testicular morphogenesis, i.e. formation of the seminiferous cords, could be dissociated from endocrine cytodifferentiation.

The histological structure of the gonads after 3 or 4 days *in vitro* suggested that serum prevented the aggregation of the Sertoli cells, and therefore morphogenesis of the seminiferous cords. Laminin and fibronectin, identified by immunohistochemical techniques, were expressed as small deposits interspersed between the Sertoli and the mesenchymal cells (Agelopoulou & Magre 1987).

The condition described after 4 days *in vitro* is transient. If the culture time in the presence of serum is prolonged for 9 days, or if at the end of the first 4 days the explants are grafted under the kidney capsule of castrated adult rats, the Sertoli cells aggregate and form seminiferous cords (A. Jost & S. Magre, unpublished results).

A second set of experiments was made with a competitor of proline, L-azetidine 2-carboxylic acid (LACA), a compound used in many embryological studies for studying the role of collagen in developing organs (lung, mammary gland, etc., references in Jost *et al.* (1985)).

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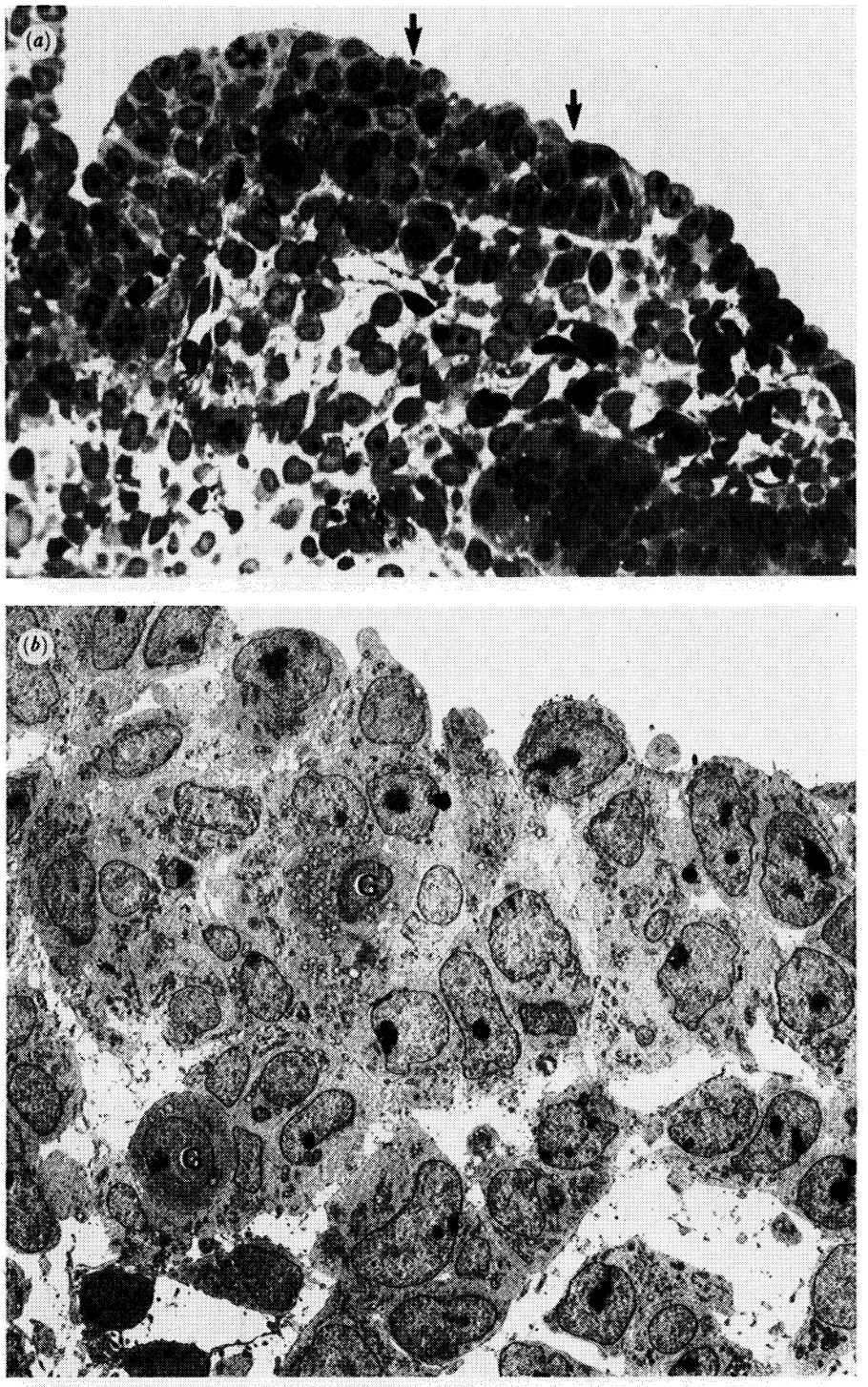

FIGURE 1. The indifferent stage of the rat gonad. Semi-thick (*a*) and thin (*b*) sections through the same gonad from a 12 day 15 h male rat foetus. The thin section illustrates the zone shown between the two arrows. The same germ cells (G) are seen in the two sections. Fixation in buffered glutaraldehyde (1% by volume). (Magn. ×600 and ×2000 respectively) (From Magre 1983.)

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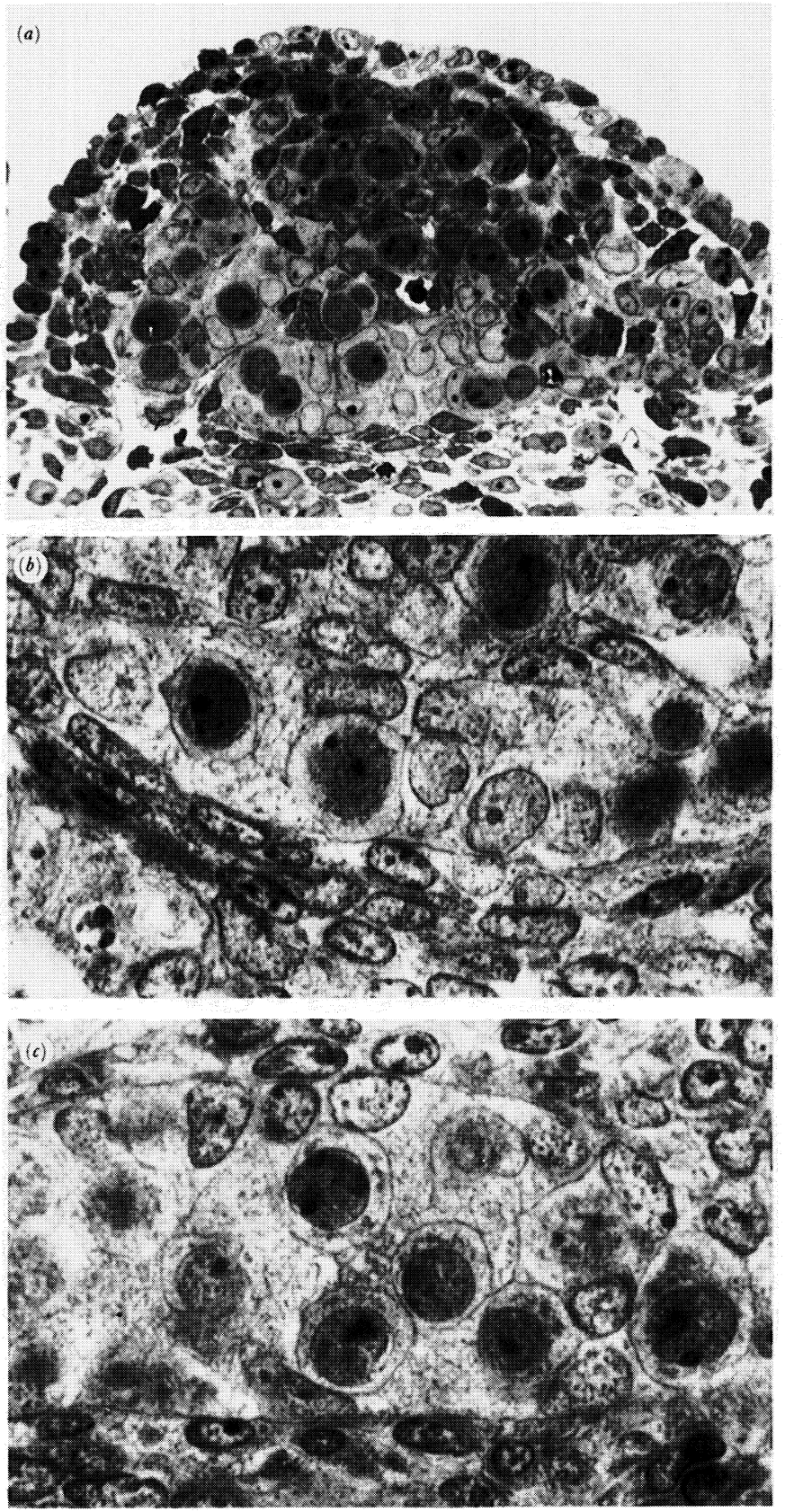

FIGURE 2. Early differentiation of the testis in the rat foetus. In (*a*) and (*b*) the first Sertoli cells surrounding some germ cells (large dark nucleus) appear in the depth of the gonad on day 13 (13 day 9 h in (*b*) and 13 day 15 h in (*a*)). (*c*) Differentiated seminiferous cord in the testis on day 14 (14 day 14 h foetus). (*a*) Semi-thick section, magn. ×600, from Magre & Jost (1980); (*b*, and *c*) Histological sections, magn. ×1400, fixed in glutaraldehyde plus picric acid, from Jost (1972).)

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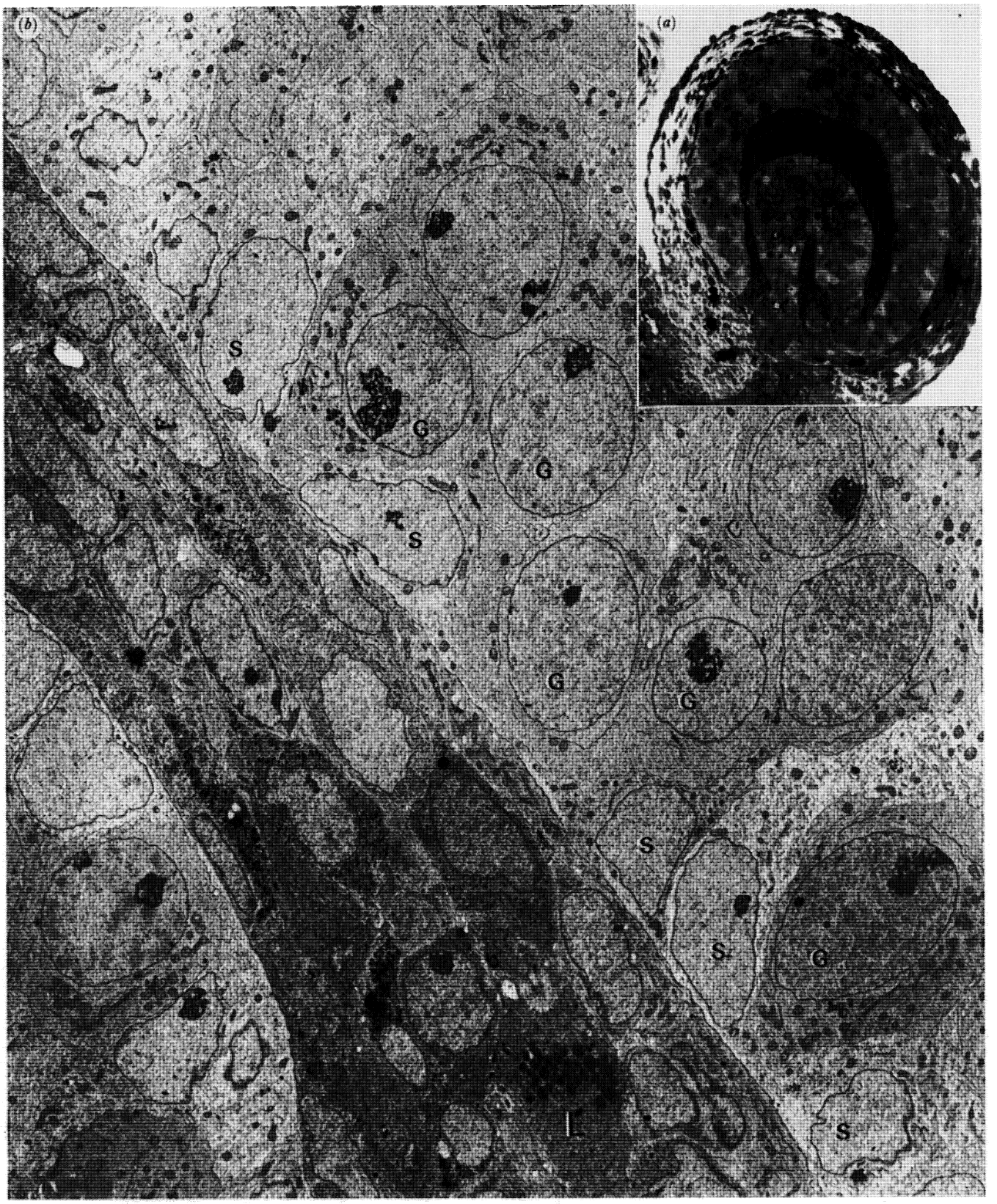

FIGURE 3. Rat testis from a 15 day 13 h foetus. Between two seminiferous cords (lower left and upper right) a dense zone of interstitial tissue is seen; the differentiating Leydig (L) cells contain lipid droplets. (G, germ cell; S, Sertoli cells; magn. ×3250). Inset: section through the same testis showing two seminiferous cords in a double arcade. The interstitial tissue is dense. (Magn. ×600.) (From Magre & Jost 1980.)

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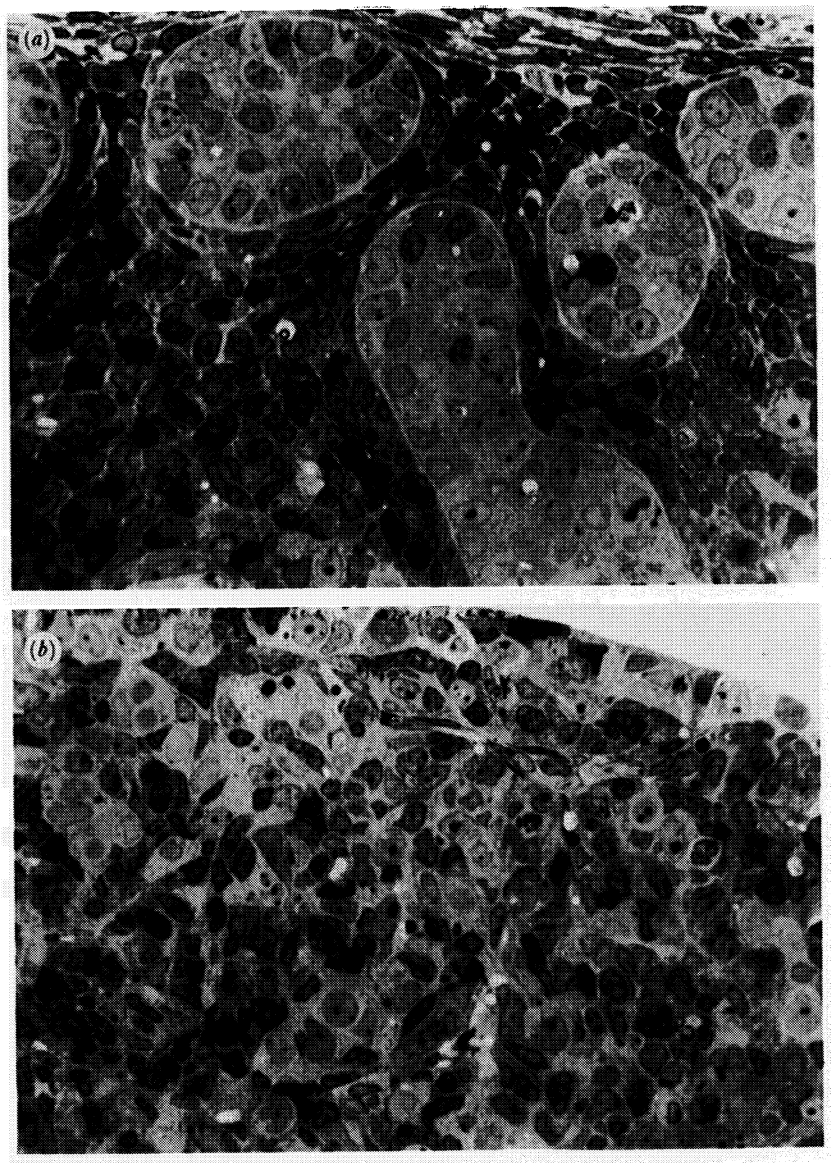

FIGURE 4. Semi-thick sections through male gonads taken from 13 day 9 h foetuses and cultured *in vitro* for 4 days. (*a*) Control gonad grown in synthetic medium (CMRL, 1066 medium). It contains well-differentiated seminiferous cords. (*b*) Gonad cultured in the same medium supplemented with foetal calf serum (15% by volume). The differentiation of seminiferous cords was prevented. (Fixation in buffered glutaraldehyde (0.5% by volume) and paraformaldehyde (20 g l^{-1}). (Magn. ×600.) (From Magre 1983.)

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A complete report of the observations will be given elsewhere. To summarize the main findings, when LACA (100 μg ml^{-1}) is added to the culture medium, well-defined seminiferous cords fail to differentiate; however, the histological appearance of the gonad differs from that obtained after the addition of serum. Many large clear cells are present and grouped into large clusters rather than being scattered throughout the gonad. The mesenchymal cells situated adjacent to these clusters do not differ from the more distant ones; they display no tendency to flatten as they do in a normal testis. Unlike with the serum-treated gonads, no laminin or fibronectin expression could be detected by immunohistochemical techniques. The endocrine cytodifferentiation of the gonads was also different. The Müllerian inhibitory activity was present, in agreement with the histological aspect of the Sertoli cells. On the contrary, only a few exceptional cells gave a weak positive response to a histochemical test of 3β HSDH activity. At the same time, the test was highly positive in the adrenal cortex (when adrenals were included in the explant). Testosterone production, if any, was below or at the limit of sensitivity of the method. Dibutyryl cAMP (10^{-3} M) added to the medium only very slightly increased testosterone release, whereas in controls it multiplies it 8-fold. The addition of 250 μg ml^{-1} L-Proline to the medium in addition to LACA permitted the formation of seminiferous cords, the normal histochemical distribution of 3β HSDH activity and testosterone secretion, as well as the response to cAMP.

A third series of preliminary studies was done with gonads from mouse foetuses (OF_1, Iffa Credo) cultured in media with or without a high concentration of a commercial preparation of α-globulin (5 mg ml^{-1} human fraction IV_1, Sigma). Previously, the α globulin fraction of human serum was found to be responsible for the effect of serum in rats (Chartrain *et al.* 1984).

Seminiferous cord formation was prevented in undifferentiated gonad primordia from 11-day-old mice cultured for 4 or 8 days in the synthetic medium with added 'α globulin'. The cordless gonads produced the Müllerian inhibitor (Picon's test on rats) but they contained almost no cells positive for the β HSDH activity. Results obtained with rat primordia showed similar absence of cord organogenesis and of steroidogenesis; the production of Müllerian inhibitor has not yet been studied.

As in the rat gonads exposed to LACA, the mouse gonads cultured in the presence of the α-globulin fraction showed failure of seminiferous cords to form, and dissociation between Müllerian duct inhibiting activity which was expressed, and 3β HSDH activity which was virtually absent.

Conclusion

The differentiation of a testis results from a cascade of multifarious morphogenetic and cytophysiological processes staggered over a prolonged developmental period. Several of these processes can be experimentally dissociated from one another. The recognition of the successive processes of cell differentiation and morphogenesis gives clues suggesting separate control mechanisms.

In humans, some genes involved in gonadal endocrine activity can be assigned to definite chromosomes. The gene coding for the anti-Müllerian hormone resides on chromosome 19 (Cohen-Haguenauer *et al.* 1987), the gene for the cholesterol side-chain cleavage enzyme (P450scc) on chromosome 15 (Chung *et al.* 1986), and the gene for 17α hydroxylase/17,20 lyase (P450c17) on chromosome 10 (Matteson *et al.* 1986).

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The same or similar genes probably also exist in the rat but their chromosomal localization is unknown. The situation created in male rat gonads cultured in the presence of serum would exemplify the expression of the three genes in the absence of seminiferous cord differentiation. Under the influence of LACA or α-globulin the formation of the cords is also prevented, and only the gene coding for the Müllerian inhibitor is expressed whereas steroidogenesis is silent.

The testis-determining gene(s) might well play no direct role in any of the cellular processes resulting in testicular differentiation, but rather 'turn on' other genes possibly residing on different chromosomes and expressed according to the particular testicular chronology.

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Discussion

Anne McLaren, F.R.S. (*MRC Mammalian Development Unit, University College London, U.K.*). Anti-Müllerian hormone (= Müllerian inhibitor) is reported to be produced not only by foetal Sertoli cells, but also by follicle cells in the adult ovary. Is it known whether all follicle cells, even those in primordial follicles, produce this hormone, or is it restricted to growing follicles?

A. Jost. AMH is not produced by follicle cells during the first few days after birth, which would suggest that it is restricted to growing follicles.

H. Sharma (71 *Barrack Road, Hounslow, U.K.*). In the role of foetal calf serum inhibiting development *in vitro*, was foetal calf serum pooled from male and female foetuses?

A. Jost. It certainly was, because we used a commercial preparation. We did other assays using serum from either male or female rats, in which the sex of the serum donor did not influence the mode of differentiation of the gonads.

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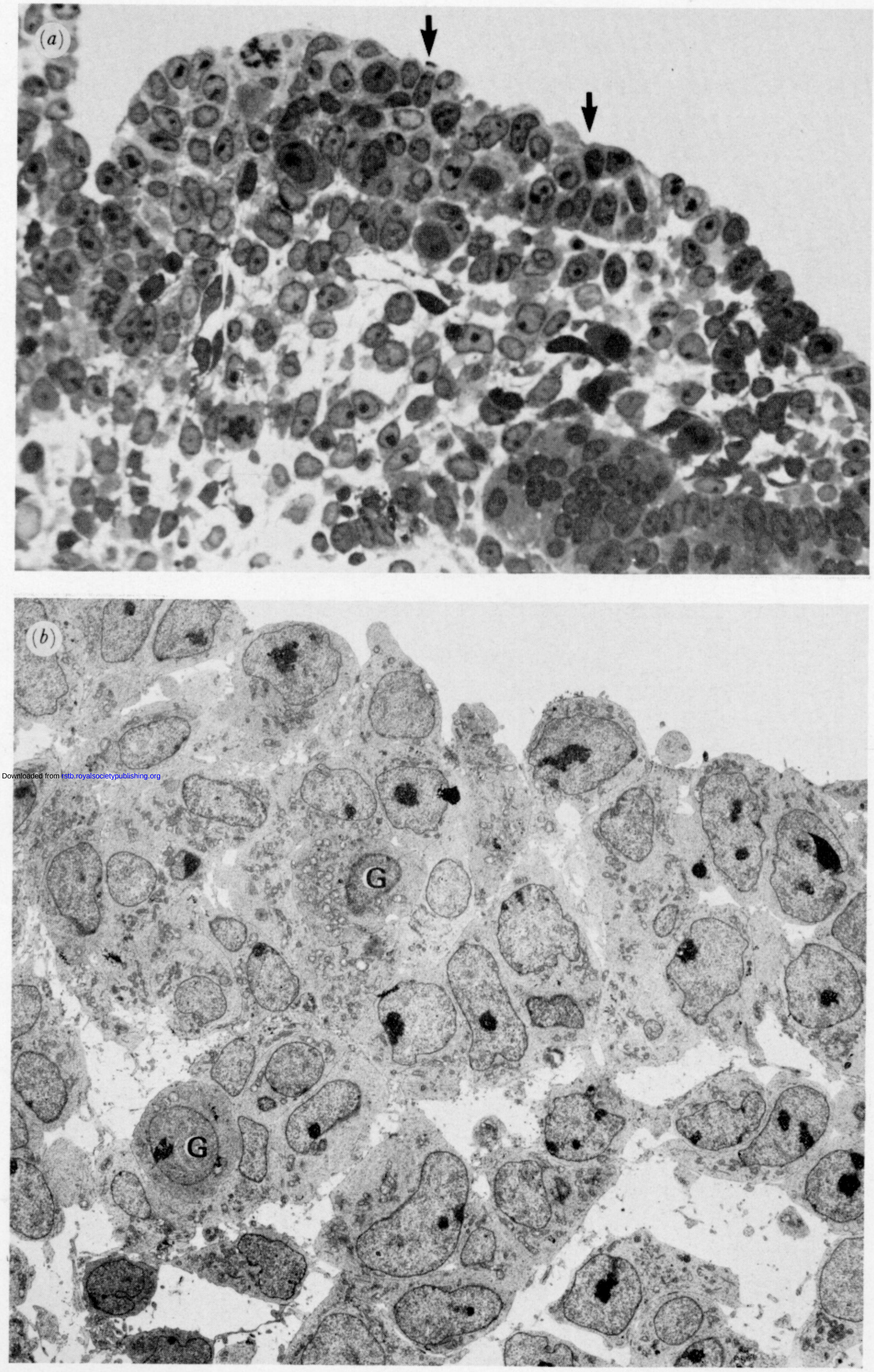

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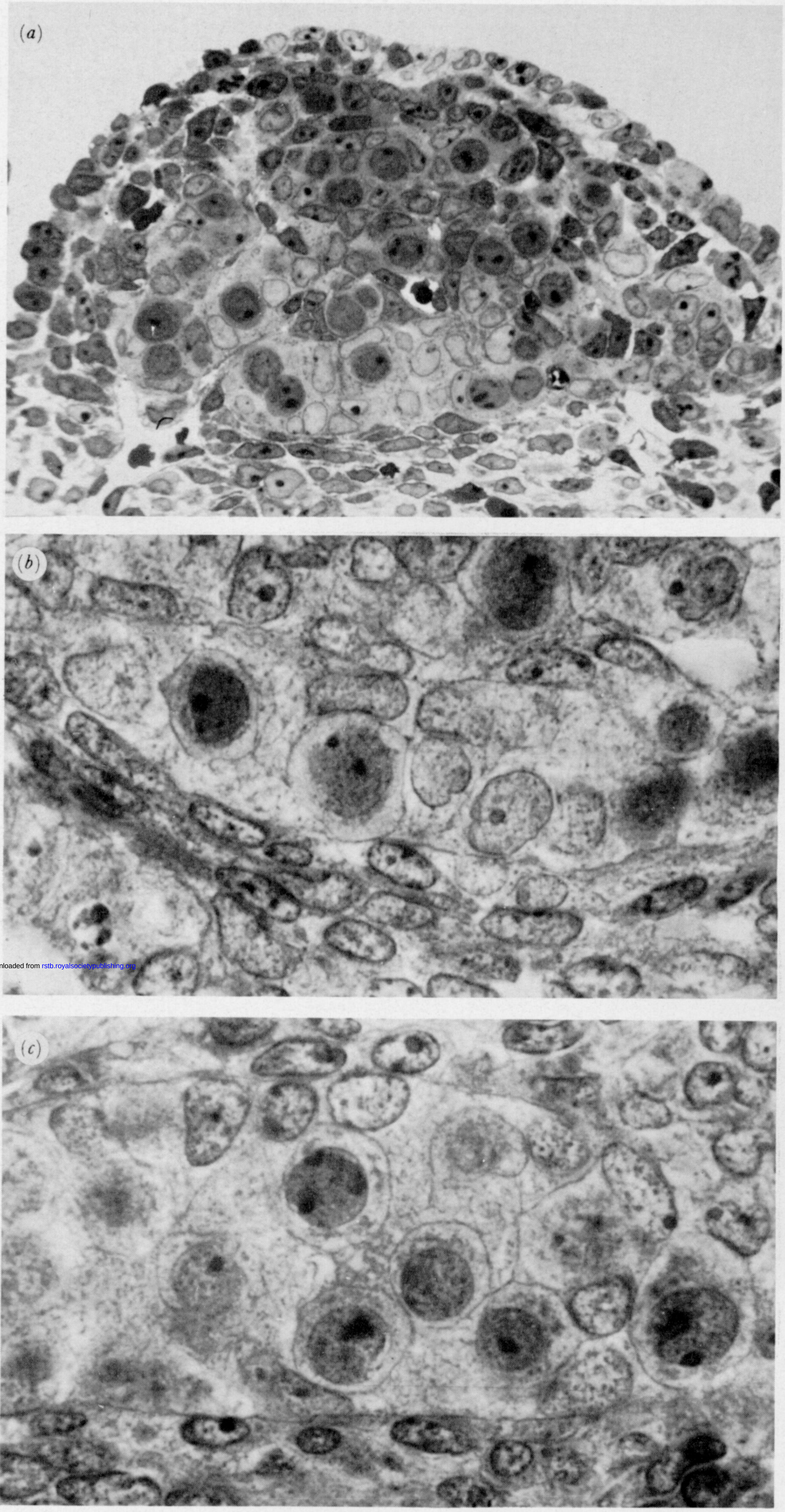

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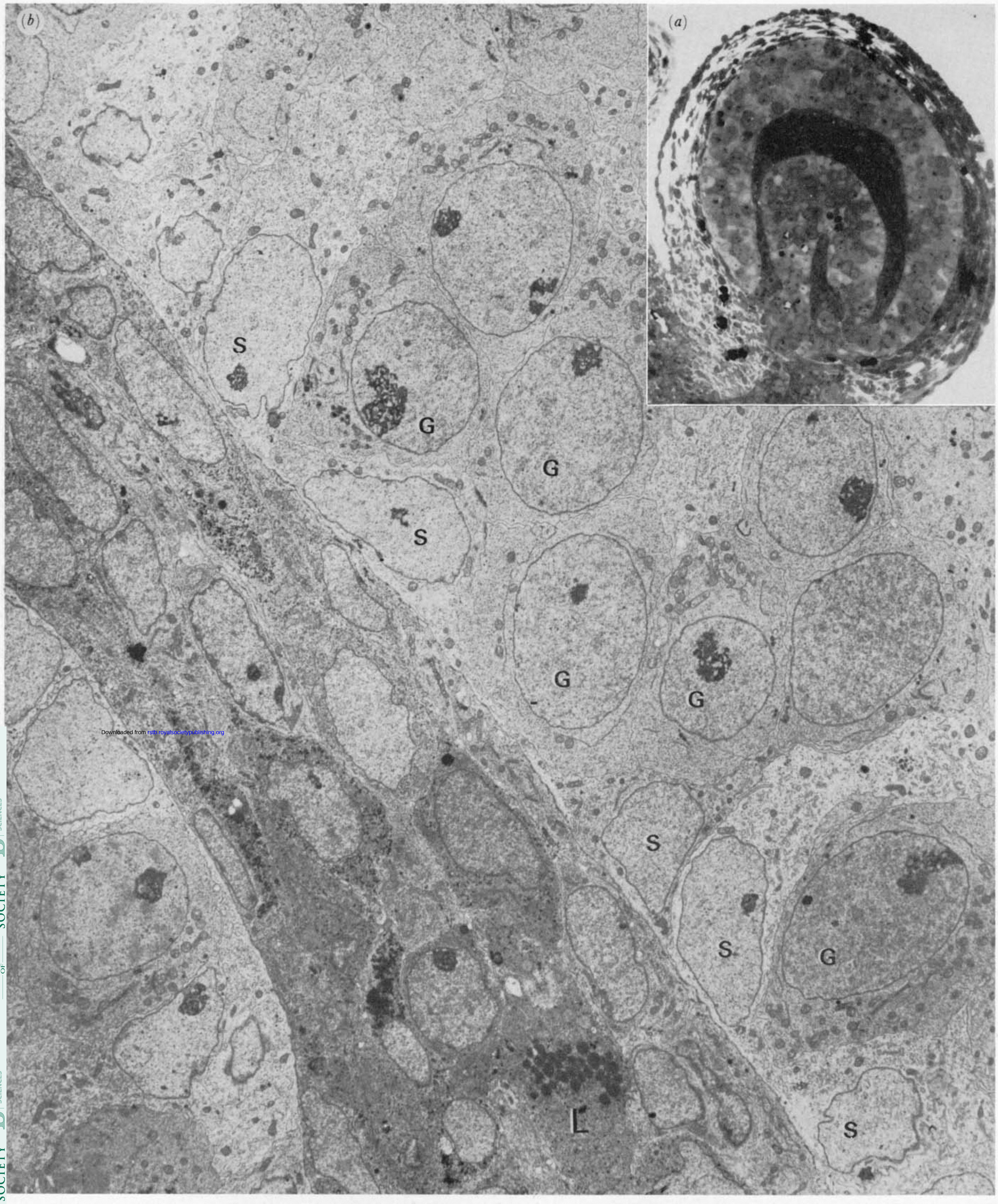

FIGURE 3. Rat testis from a 15 day 13 h foetus. Between two seminiferous cords (lower left and upper right) a dense zone of interstitial tissue is seen; the differentiating Leydig (L) cells contain lipid droplets. (G, germ cell; S, Sertoli cells; magn. ×3250). Inset: section through the same testis showing two seminiferous cords in a double arcade. The interstitial tissue is dense. (Magn. ×600.) (From Magre & Jost 1980.)

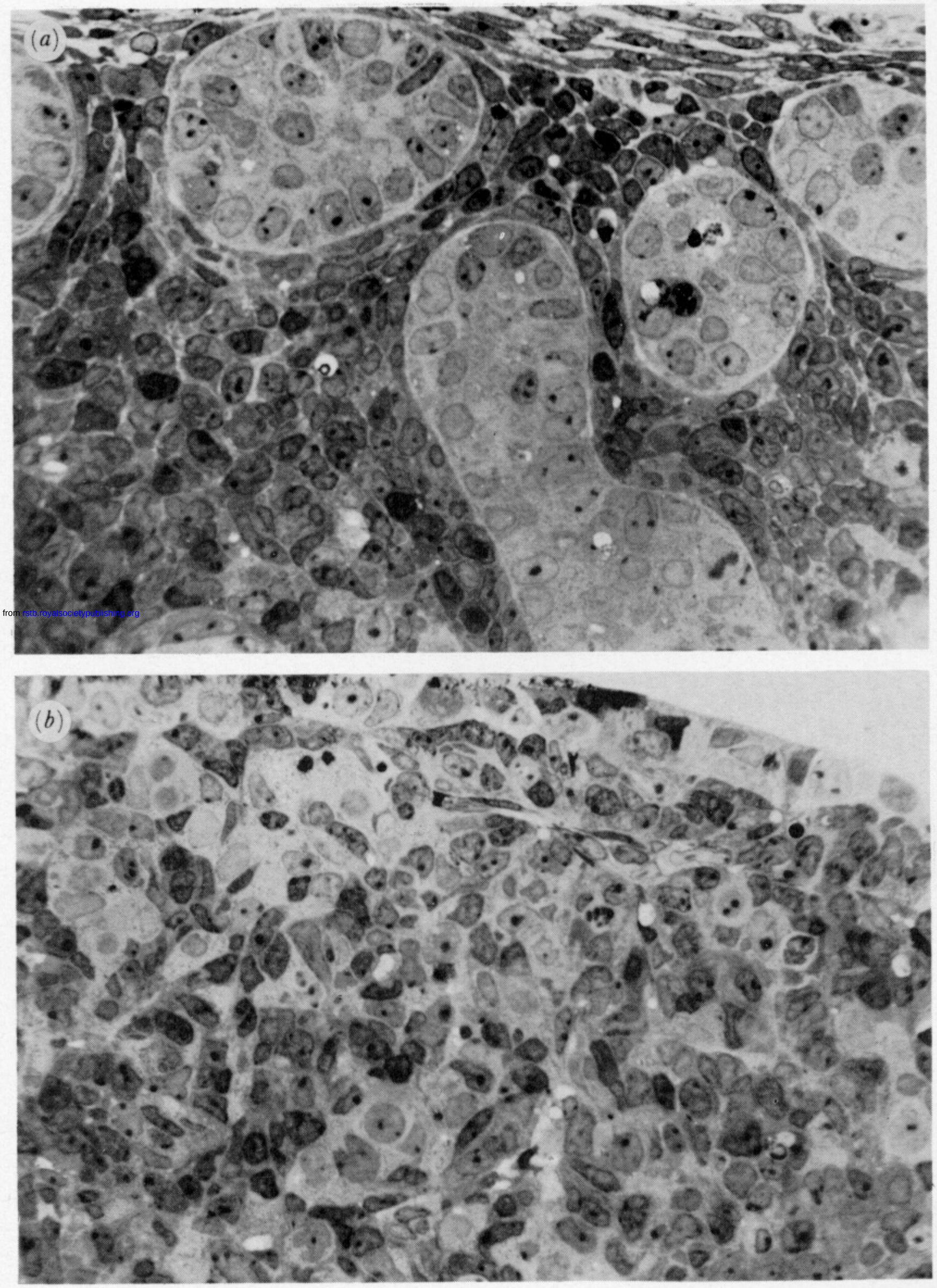

FIGURE 4. Semi-thick sections through male gonads taken from 13 day 9 h foetuses and cultured *in vitro* for 4 days. (*a*) Control gonad grown in synthetic medium (CMRL, 1066 medium). It contains well-differentiated seminiferous cords. (*b*) Gonad cultured in the same medium supplemented with foetal calf serum (15% by volume). The differentiation of seminiferous cords was prevented. (Fixation in buffered glutaraldehyde (0.5% by volume) and paraformaldehyde (20 g l^{-1}). (Magn. ×600.) (From Magre 1983.)