BIOLOGY OF REPRODUCTION 9, 500–532 (1973)

Comparative Observations on Intertubular Lymphatics and the Organization of the Interstitial Tissue of the Mammalian Testis[1]

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Accepted May 21, 1973

The interstitial tissue of the testis has been studied in some 14 mammalian species and compared with respect to the abundance of Leydig cells and loose connective tissue, the degree of development and location of the interstitial lymphatics and their topographical relationship to the endocrine cells and to the seminiferous tubules.

Marked differences were found among the species studied but in general they could be assigned to one of three patterns of organization: (1) Species with a relatively small volume of Leydig cells and very little connective tissue stroma but provided with extensive peritubular lymphatic sinusoids or lymph spaces which occupy a large part of the intertubular area. (2) Species with clusters of Leydig cells scattered in an abundant, loose, edematous connective tissue which is drained by a lymphatic vessel, centrally or eccentrically placed in each intertubular area. (3) Species having very abundant, closely packed Leydig cells that occupy nearly all the enlarged intertubular areas, but with little connective tissue and very few interstitial lymphatics of small size. The possible physiological implications of these differing patterns of interstitial tissue organization are discussed.

The literature on the comparative aspects of spermatogenesis and sperm structure is voluminous but there have been few comparative studies on interspecific variations in the organization of the interstitial tissue of the testis. Indeed much of the basic information essential for valid correlation of interstitial tissue structure and function is still lacking. This is due, in part, to the fact that traditional methods of specimen preparation were subject to major artifacts of shrinkage and swelling that seriously distorted the relationships of the interstitial components to each other and to the tubules.

Especially contradictory is the literature dealing with the lymphatics of the testis. Several of the early investigators described a network of small lymph vessels between the seminiferous tubules which were said to drain via larger lymphatic vessels in the septula and tunica albuginea (Gerster, 1876; Hasumi, 1930). Others were unable to identify intertubular lymphatics and reported instead, lymph-filled interstitial spaces which, in larger species, were believed to drain into open lymph vessels located in the septula testis (Ludwig and Thomsa, 1861, 1862). The majority of investigators who have addressed themselves to this question in the past two decades have denied the existence of interstitial

[1] Supported in part by Contract NIH-69-2107 from the Center for Population Research, and in part by GM-02344 from Institute of Child Health and Human Development.

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lymphatic vessels and have described the lymphatic system of the testis as beginning either in the septula (Most, 1899; Jamieson and Dobron, 1910; Grau and Karpf, 1963; Staudt and Wenzel, 1965; Frey, 1963; Karpf and Taher, 1965) or in the tunica albuginea (Renyi-Vamos, 1955, 1956; Brzezinski, 1963).

The subject has been reopened as a result of the recent demonstration of parenchymal lymphatics by retrograde gelatin injection in bull (Hundeiker and Keller, 1969) and in the human testis (Hundeiker, 1969) and by electron microscopic studies of perfused guinea pig and chinchilla testes (Fawcett *et al.*, 1969). The latter studies revealed an extensive system of thin-walled lymphatic sinusoids surrounding the seminiferous tubules. The Leydig cells were clustered around small centrally located blood vessels while the lymphatic sinusoids occupied the periphery of the interstitial areas. The endocrine cells were thus found to be interposed between the blood vascular system and the lymph. Some possible physiological implications of this arrangement were discussed in a previous paper (Fawcett *et al.*, 1970).

The present study extends these observations to a number of laboratory and domestic animals (rat, mouse, opossum, monkey, ram, boar, and bull) and to several feral East African species (hyrax, elephant, wart-hog, zebra, and mole rat). Marked interspecific variations were found in the abundance of Leydig cells, the amount of intertubular connective tissue, and the location and degree of development of the lymphatics. The variations observed were not explainable in terms of phylogeny inasmuch as striking differences were often found between members of the same Order and significant differences within the same Family. In directing attention to these variations in the histological organization of the interstitial tissue, it is our purpose to discourage extrapolation to other species from experimental results obtained in laboratory rodents and to encourage others to take these differences into account in their interpretation of the physiology of the mammalian testis.

MATERIALS AND METHODS

The observations are based upon examination of testes from approximately 15 guinea pigs, 4 rats, 2 mice, 2 rams, 5 domestic boars, 1 Friesian bull, 2 North American opossums, and 4 rhesus monkeys. The boars were of an inbred strain (Pitman-Moore) of miniature pigs supplied by the Vita-Vet Laboratories, Marian, Indiana. The rams were of Suffolk breed purchased from local farmers in Massachusetts. The East African material included 3 wart-hogs (*Phacochoerus aethiopicus*), 4 naked mole rats (*Heterocephalus glaber*), 1 elephant (*Loxodonta africana*), 2 conies (*Heterohyrax brucei*), 2 zebra (*Equus burchelli*) collected in Kenya.

The testes of all domestic and common laboratory species were fixed by vascular perfusion. Wherever possible, specimens from wild species were similarly fixed by perfusion but difficulties of collecting in the field sometimes made it necessary simply to fix small blocks of tissue by immersion. Perfusions were carried out by a modification of the procedure described by Christensen (1965). The spermatic cord was clamped and the testis removed from the scrotum. A needle of appropriate size affixed to a length of polyethylene tubing was introduced into the spermatic artery or one of its branches on the surface of the testis and physiological salt solution was allowed to flow through the tubing by gravity from a reservoir at a height not exceeding the estimated blood pressure of the species in centimeters of water. As soon as flow was established, the testis blanched, and the hemostat was removed and the pampiniform plexus incised to permit venous outflow. After 20–30 min, perfusion was terminated and the hardened testis was cut into thin transverse slices and blocks 1.5 mm square were cut from these with a sharp razor and fixed an additional hour by immersion in fixative of the same composition. In mice and rats, because of their smaller size, perfusion was carried out retrograde through the abdominal aorta with a ligature above the renal arteries and with the testes *in situ*.

The fixatives employed were *s*-collidine buffered 5% glutaraldehyde or a mixture of picric acid, paraformaldehyde and glutaraldehyde (Ito and Karnovsky, 1968) based upon a method described by Stefanini, De Martino, and Zamboni (1967). Tissues were post-fixed an hour in 1% buffered osmium tetroxide, then dehydrated in increasing concentrations of ethyl alcohol and embedded in Epon or Epon–araldite mixture.

Sections 0.5–1 μm thick were cut and stained

for light microscopy with toluidine blue in borax (Richardson *et al.*, 1960). These sections, examined with the light microscope, provided the resolution required for most of the observations on the topography and histological organization of the interstitial tissue. Sections were photographed with a 40× oil immersion objective on a Zeiss microscope.

Where higher resolution was required, thin sections with yellow interference colors were cut from the same blocks with a Sorvall MT-1 ultramicrotome using a diamond knife. Such sections were stained with uranyl acetate and lead citrate (Venable and Coggeshall, 1965) and examined with RCA-3G or Phillips 200 electron microscopes.

OBSERVATIONS

Some of the confusion about the intertubular lymphatics of the testis appears to be semantic. It may therefore be helpful to define, at the outset, our criteria for recognition of lymphatics at the light and electron microscopic levels, and to explain our use of the terms *lymphatic vessel, lymphatic sinusoid,* and *lymph space.* The identifying characteristics of lymphatics upon which histologists have traditionally relied are (1) variable caliber and large lumen relative to the thickness of their wall, (2) a more irregular cross-sectional outline than blood capillaries, (3) an extremely attenuated lining endothelium, (4) lack of pericytes or recognizable adventitia and (5) absence of blood cells in the lumen. In thin plastic sections of material fixed by vascular perfusion, one has the additional criterion (6) that the lumen of lymphatics contains a uniform grey precipitate of the proteins of the lymph, whereas in small blood vessels that might otherwise be confused with them, the plasma proteins and blood cells have been washed out in the perfusion and the lumen appears entirely empty. The higher resolution of electron microscopy provides additional identifying criteria that have been detailed by Fraley and Weiss (1961) and Leak and Burke (1966, 1968). (1) The lining endothelium lacks a basal lamina and projections of the cell both into the lumen, and from the abluminal side, give the lymphatic endothelium more irregular surface contour than that of the blood capillaries and venules. (2) The endothelial cell junctions are often extensively overlapping and the apposing membranes are loosely adherent with only occasional punctate specializations for attachment.

We define here as a *lymphatic vessel* any endothelium-lined tubular channel in the interstitial tissue which has the above characteristics. We describe as *lymphatic sinusoids* more spacious, endothelium-lined, intercommunicating cavities that are not tubular but are highly irregular and variable in shape because their wall conforms to the contours of the spaces they occupy and of the structures they surround. Thus, in the case of the rodent testis, the form of the seminiferous tubules and the disposition of the blood vessels with their associated Leydig cell clusters determine the configuration of a labyrinthine system of lymphatic sinusoids that occupy all of the remaining interstitial space. It now appears that what earlier authors referred to as *lymph spaces* are, in fact, sizable intercommunicating cavities filled with protein-rich lymph but only partially lined by endothelium. Their lumen is continuous with the extracellular fluid phase of the matrix of the adjacent areolar connective tissue. Their limits may be poorly defined in some areas while in others they may have a discontinuous lining of endothelial cells that gradually becomes continuous where the spaces become confluent with the lumen of a typical lymphatic vessel. These structures could just as appropriately be called *discontinuous sinusoids.* The distinctions between these elements will become more clear in the description of the interstitial tissue in various species which follows.

Guinea Pig (Cavia porcella)

The organization of the interstitium of the guinea pig has been described elsewhere (Fawcett *et al.*, 1969, 1970) and need only be briefly reviewed here as a

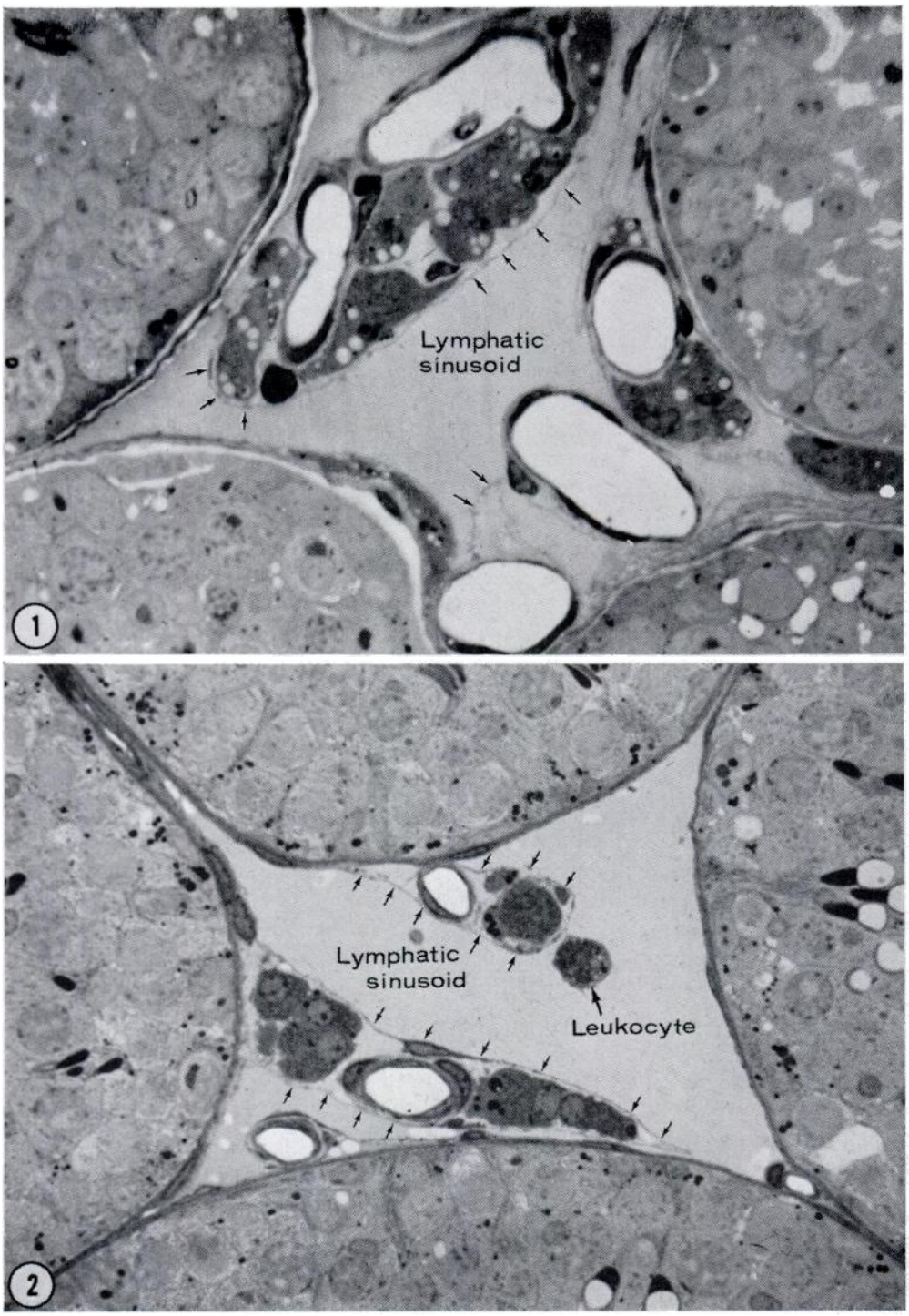
basis for comparison with other species. The clusters of Leydig cells are intimately associated with small blood vessels that tend to be centrally located in the angular intertubular areas. The blood vessels together with their associated Leydig cells are invested by an extremely thin endothelium which can just be resolved in sections viewed with the light microscope (see at arrows in Figs. 1 and 2). However, this sheet of endothelium is clearly visualized in electron micrographs. A cytologically similar layer is closely applied to the peritubular layer of epithelioid contractile cells (myoid cells) surrounding the seminiferous tubules. The peritubular investment of endothelium is always readily identifiable in electron micrographs but, because it is so closely juxtaposed to the peritubular myoid cells, it is not always resolved as a separate layer with the light microscope. These perivascular and peritubular layers of endothelium bound a system of irregularly shaped intertubular lymphatic sinusoids that fill nearly all of the intertubular area not occupied by Leydig cells and blood vessels. The sheet of thin, flattened cells that covers these latter structures is referred to hereafter as the *visceral layer* of lymphatic endothelium while the layer applied to the tubules is called the *parietal layer* by analogy with the visceral and parietal peritoneum.

Two apposed layers of the visceral lymphatic endothelium frequently form thin mesentery-like structures that loosely attach the endocrine-vascular complex of each interspace to a neighboring tubule (Figs. 2 and 3). Broader areas of attachment of the Leydig cell clusters to the walls of tubules are relatively rare. Interstitial connective tissue is very sparse in this species, being represented by occasional fusiform cells and small bundles of collagen fibrils around the vessels, among the Leydig cells and in the mesentery-like attachments to the tubules. In specimens of testis from animals in which saline has been injected interstitially prior to perfusion (Fig. 3), occasional small gaps are seen in the visceral endothelium lining the distended sinusoids. These may or may not be a consequence of the treatment. We regard the endothelium of the lymphatic sinusoids in the guinea pig as essentially continuous but accept the possibility that very small discontinuities in the visceral layer may occur normally. The parietal layer always appears to be continuous.

The clear spaces seen around and between seminiferous tubules in routine histological sections of rodent testis have long been regarded as shrinkage artifacts and no physiological significance was attributed to them. Improved methods of specimen preparation for light and electron microscopy have now provided a more satisfactory interpretation of these spaces. In the guinea pig, the seminiferous tubules are everywhere surrounded by fluid-filled lymphatic sinusoids that long escaped attention because they are invariably disrupted and their contents are extracted in routine histological preparations. It is now evident that the Leydig cells are interposed between the blood vascular system on one side and the lymphatic labyrinth on the other. These cells are not functionally polarized and undoubtedly they release androgen into both vascular systems. The peritubular lymphatic sinusoids therefore must play an important role in distribution of hormones and metabolites to the seminiferous epithelium. A similar organization of the interstitial tissue has been observed in the chinchilla and probably will be found in other hystricomorph rodents.

Rat (Rattus rattus) *and Mouse* (Mus musculus)

Upon superficial examination, the general organization of the interstitium of the rat testis appears very similar to that of the guinea pig (Fig. 4). On closer inspection, however, a significant difference is detectable even at the light microscope level. Whereas in the guinea pig testis, a visceral layer of endothelium is clearly visi-



FIGS. 1 and 2. Photomicrographs of interstitial areas from guinea pig testis. The Leydig cells are closely clustered around the blood vessels. A thin layer of endothelium can be seen (at the arrows) surrounding the Leydig cell clusters and vessels. A similar layer is closely applied to the peritubular contractile cells. The bulk of the intertubular area is occupied by an extensive lymphatic sinusoid. Magnification 542.5×.

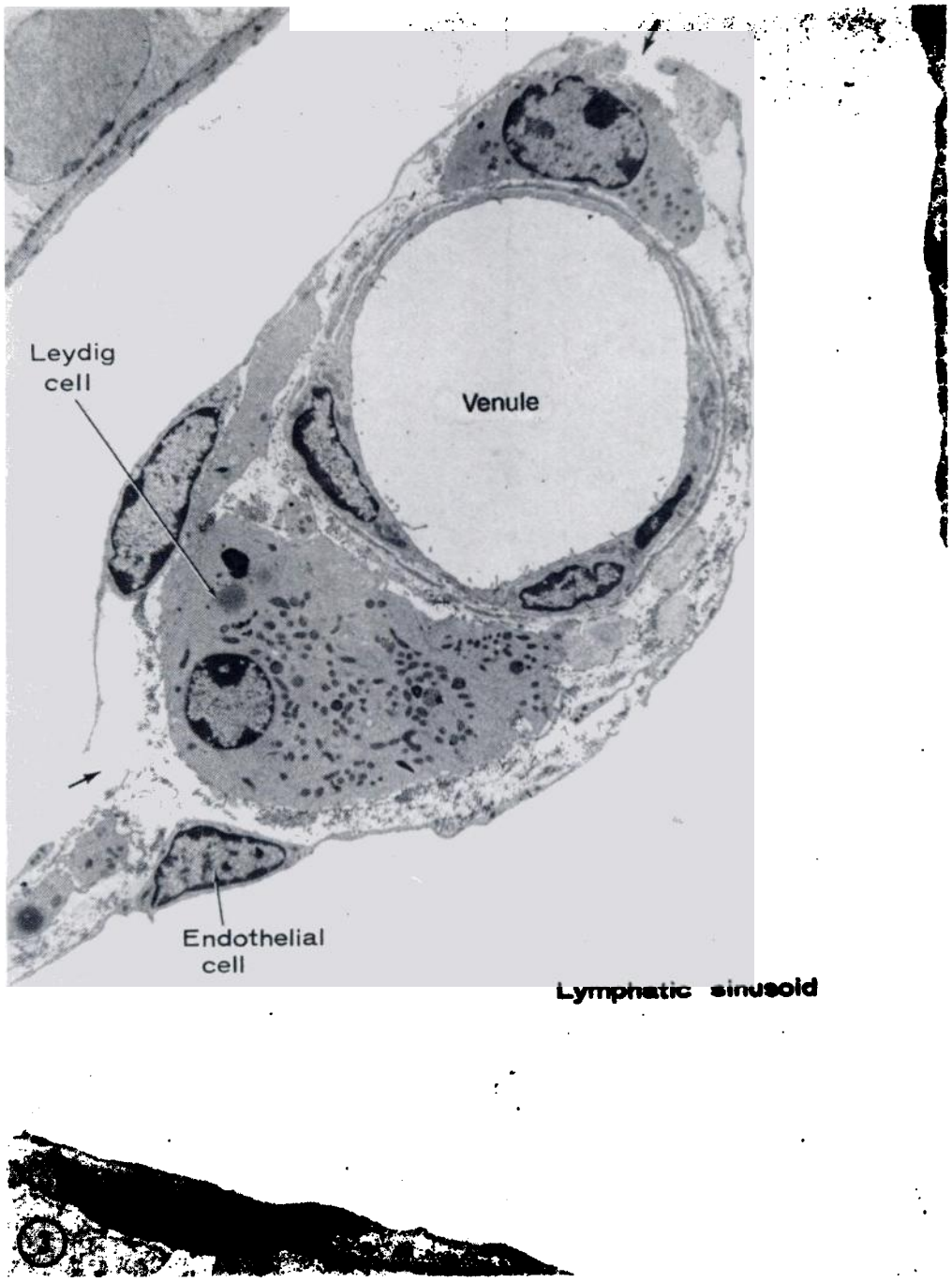
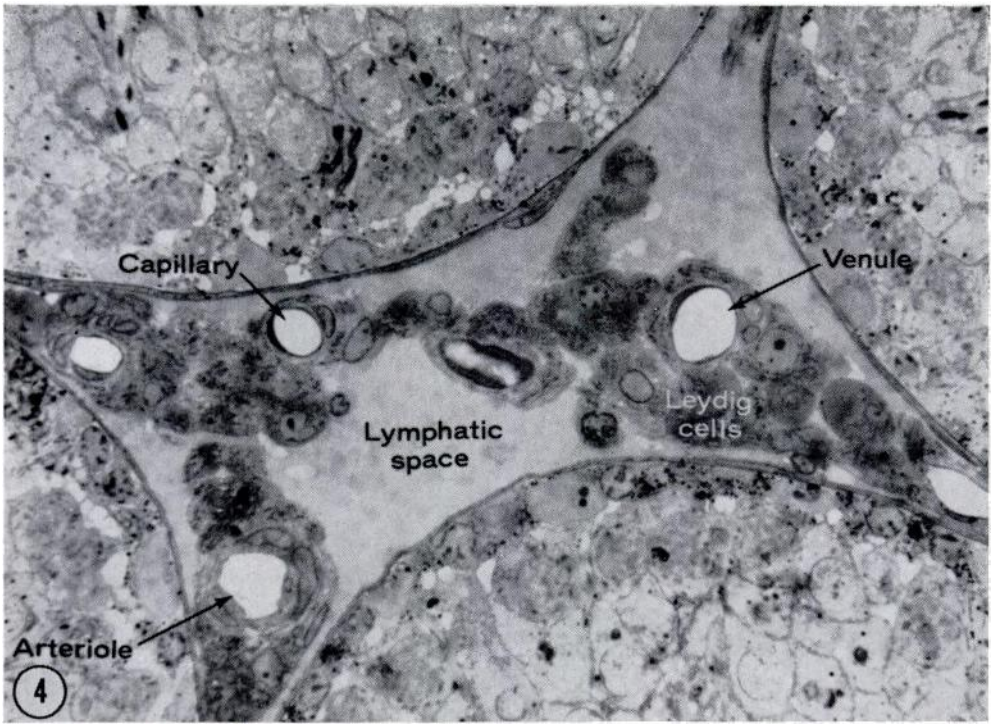


FIG. 3. Electron micrograph of a triangular intertubular area from guinea pig testis. Notice that the Leydig cells and the blood vessel with which they are associated are covered by extremely thin flattened cells that form a nearly continuous endothelium. This continues over a narrow mesentery-like structure at the lower left to become continuous with a similar layer closely adherent to the peritubular contractile cells of the seminiferous tubules. The Leydig cells are thus interposed between the blood vessel and an extensive lymphatic sinusoid. Saline had been injected interstitial into the testis shortly before perfusion. This protein-rich lymph is therefore replaced and the width of the lymphatic sinusoid slightly exaggerated. The discontinuities of the visceral endothelium at the arrows may be a normal occurrence or in this case, may have been induced by the saline injection. Magnification 3060×.



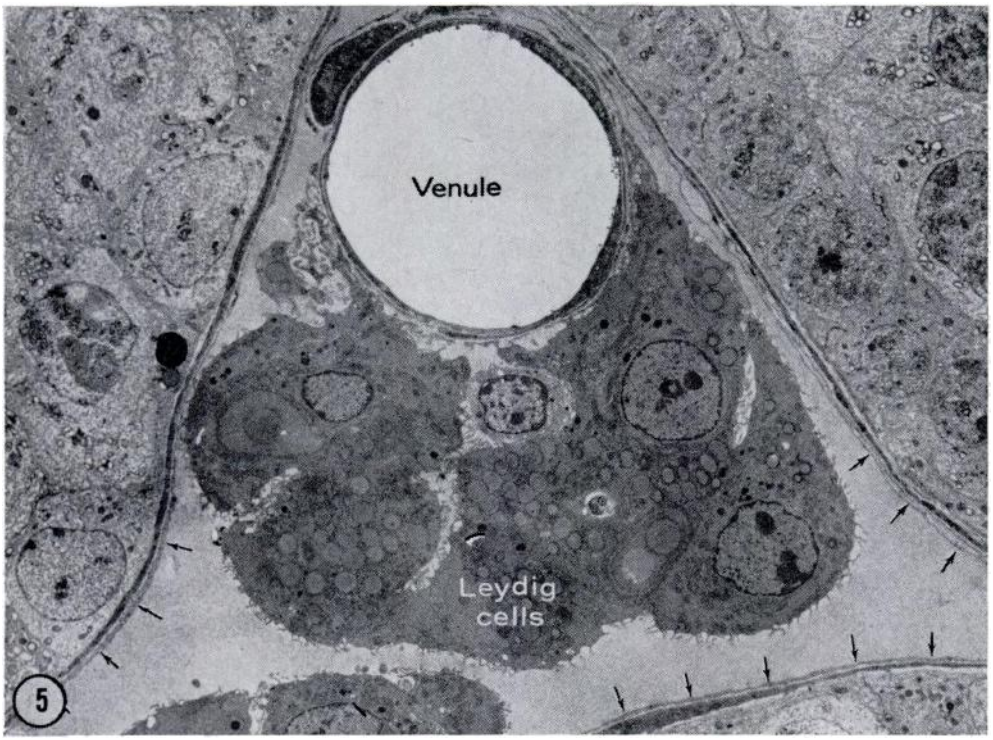


FIG. 4. Photomicrograph of a typical interstitial space from rat testis. The Leydig cells are closely associated with the blood vessels and surrounded by a lymph-filled space. In contrast to the guinea pig, a visceral layer of endothelium cannot be seen around the Leydig cell clusters. Magnification 585×.

FIG. 5. A low power electron micrograph of an interstitial area from rat testis. A parietal layer of endothelium is closely applied to the myoid layer of the seminiferous tubules (at arrows), but the microvillous surface of the Leydig cells is directly exposed to the lymph. The Leydig cells are actually within a large intertubular lymphatic sinusoid. Magnification 2250×.

ble around the vessels and Leydig cell clusters, such a layer is rudimentary in the rat and only occasionally seen with the light microscope (Compare Fig. 4 and Fig. 1). Electron micrographs of perfused rat testis confirm the suspicion that a continuous sheet of endothelium is lacking over a large part of the surface of the interstitial cell clusters. The microvillous surface of the glandular cells is bathed directly by the lymph (Figs. 5 and 7). The Leydig cells contain an abundance of lipid and occasional membranous whorls (Fig. 5). Where occasional flattened cells are found on the exterior of the Leydig cell–blood vessel complex, they have the same appearance and relationship to the underlying structures as described above for the visceral endothelium in the guinea pig testis (Fig. 7). A parietal layer of endothelium is present and does form a continuous covering on the outer aspect of the seminiferous tubules and in the narrower intertubular clefts where there are no blood vessels or Leydig cells. There the opposing surfaces of neighboring tubules are both lined by endothelium and the intervening space is filled with fine-grained grey precipitate of the protein of lymph (Fig. 6). Such areas are indistinguishable from intertubular lymphatic sinusoids of guinea pig.

Thus, despite a basic similarity in the topography of the interstitial tissue of these two species, there is a significant difference in that the visceral layer of endothelium is largely absent in the rat and a large part of the surface of the islands of endocrine cells is directly exposed to the lymph without an intervening layer of endothelium.

The situation in the mouse is rather similar to that in the rat. The visceral endothelium may be somewhat better developed (Fig. 8) but there are still many areas in which the glandular cells are directly exposed to the lymph (Fig. 9).

The fluid-filled intertubular cavities in the rat and mouse conform to earlier definitions of a *lymphatic space* for, in the absence of a visceral layer of endothelium, the lymph is continuous with the fluid in the interstices among the Leydig cells and perivascular connective tissue. However, the basic topographic similarity of rat and guinea pig is probably better conveyed by describing these as *lymphatic sinusoids with discontinuous visceral endothelium.*

In fairness to Ludwig and Thomsa (1862) and other predecessors in this field of inquiry, it is very easy to see how the organization of the interstitium in the rodents could lead to the conclusion that the interstitial capillaries were surrounded by lymph spaces without walls of their own but bounded by the walls of the seminiferous tubules and the blood vessels. Clearly if the parietal endothelium was preserved in the preparations available to these investigators, it was not seen or not recognized as such. It is equally understandable, in retrospect, that Brzezinski (1963) studying the guinea pig reported "intertubular spaces" filled with PAS-positive material that he called "primary lymph" because it seemed to him not to be contained within lymphatics. The primary lymph he assumed was "actively transported" from the "spaces" across the endothelium of closed lymphatics in the tunica albuginea.

Monkey (Macaca mulatta) *and Man* (Homo sapiens)

The organization of the interstitial tissue of the primate testis is very different from that of rodents. In the macaque, the intertubular spaces are filled with a very loose connective tissue containing abundant interstitial fluid and numerous fusiform or branching cells with long slender processes. These are presumed to be primitive fibroblasts (Fig. 12) or possibly cells with the developmental potentialities of mesenchymal cells. The lipid-rich Leydig cells occur singly, in rows, or in clumps of varying size. The clustering of these around blood vessels is not as obvious or as intimate as in rodent testis. Indeed some of the clusters and many individual Leydig

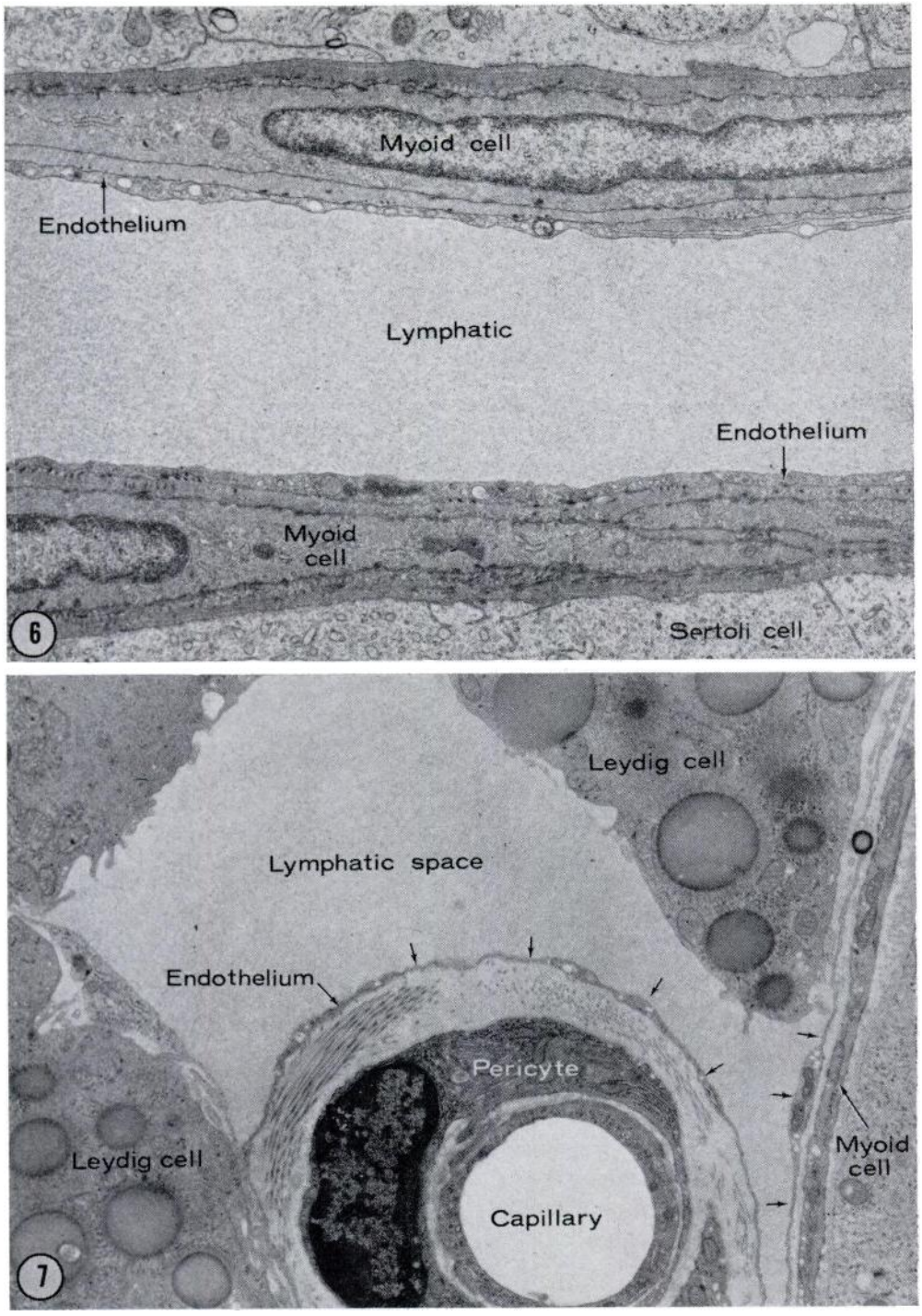


Fig. 6. An electron micrograph of a lymphatic sinusoid between two seminiferous tubules of rat testis. The parietal layer of endothelium is closely adherent to the myoid cell layer. Magnification 1020×. (Micrograph courtesy of Dr. Winston Anderson.)

FIG. 7. Electron micrograph of rat interstitial tissue. A layer of endothelium is visible adjacent to the peritubular contractile cell layer at the lower right, and over the capillary and associated collagen in the lower half of the figure. Notice however that the Leydig cells are directly exposed to the lymph without an intervening layer of visceral endothelium. Magnification 7140×. (Micrograph courtesy of Dr. R. Vitale-Calpe.)

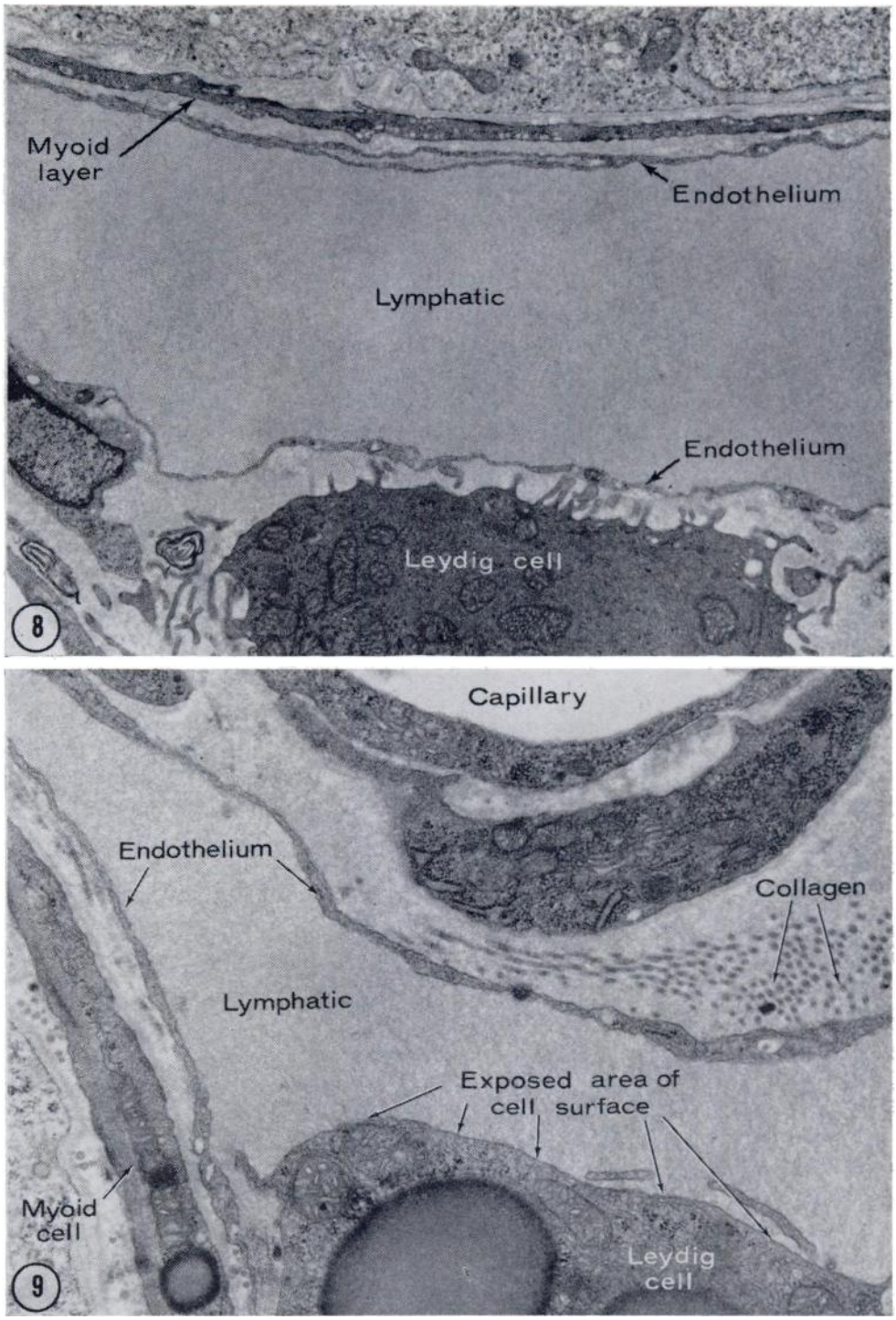


FIG. 8. Electron micrograph of mouse interstitial tissue. A peritubular lymphatic is shown with parietal layer adjacent to the tubule above and a visceral layer of endothelium overlying the Leydig cells below. Magnification 9900×. (Micrograph by R. Vitale-Calpe.)

FIG. 9. Mouse interstitial area. Notice the endothelium adjacent to the tubule at the left and around the capillary above but the Leydig cell in the lower part of the figure is not covered by endothelium and hence is within a lymphatic space. Magnification 12600×. (Micrograph by R. Vitale-Calpe.)

cells are some distance from the nearest capillary or venule. Peritubular lymphatic sinusoids are not found in primates. Instead, lymphatic vessels, often of fairly large size and variable cross-sectioned outline are quite common. These are centrally or eccentrically located in the intertubular areas (Figs. 10–12).

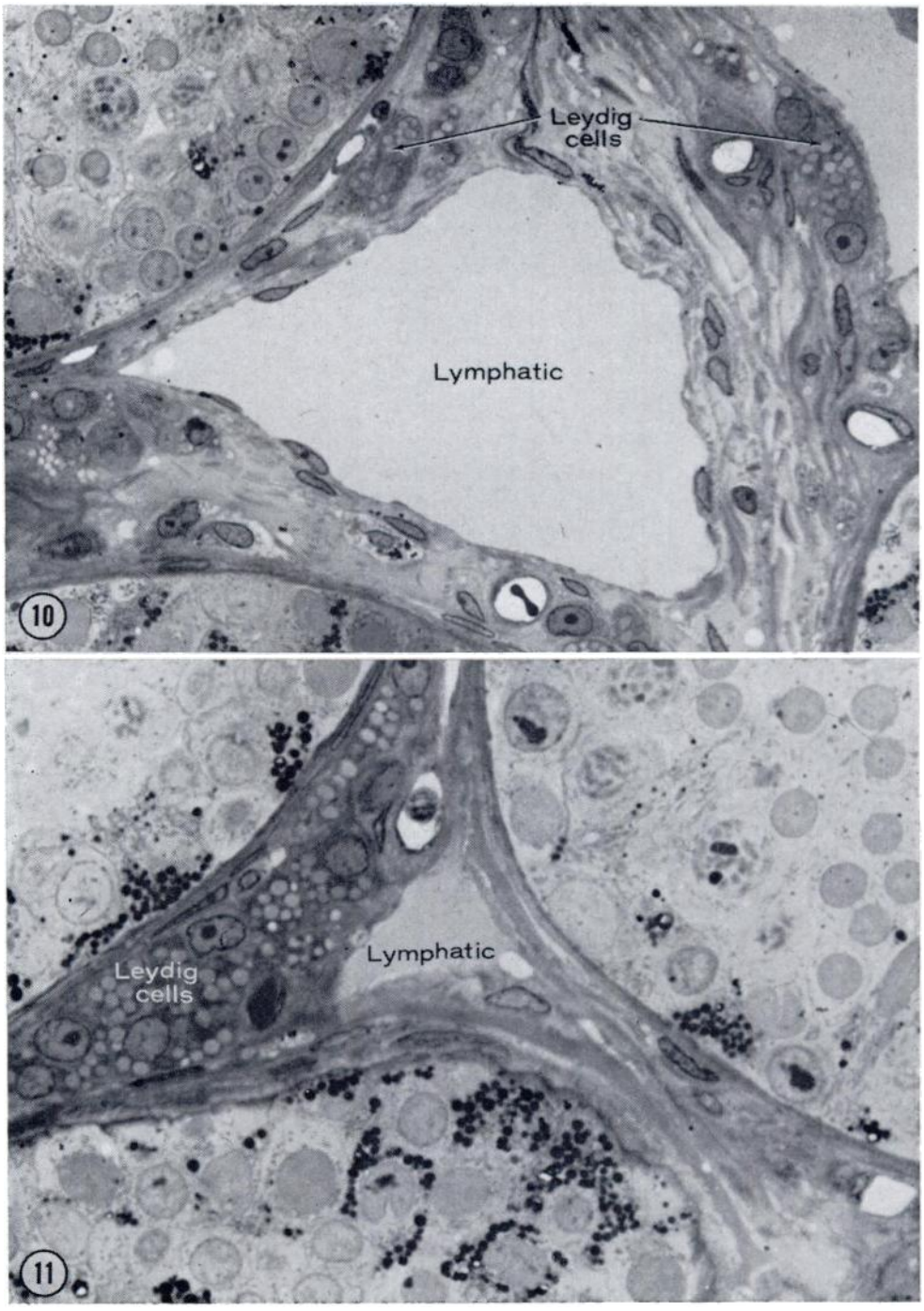
In electron micrographs, the loose connective tissue is seen to contain considerably more collagen than is found in laboratory rodents and its interstices are filled with a protein precipitate of moderate density. The contents of the lymphatics are sometimes very dark. Not infrequently, the density of the interstitial fluid is greater than that of the lymph as though there were a significant difference between the two fluids in the concentration of protein or of some other osmiophilic component. In some areas, the interstitial fluid around and between the Leydig cells is very dark (Figs. 14 and 15). These apparent concentration differences between the various tissue compartments pose interesting and puzzling physiological questions.

The interstitial tissue of the human testis is similar to that of the rhesus monkey. The intertubular areas are perhaps somewhat more spacious and the loose edematous connective tissue more abundant (Fig. 13) but the topography is basically the same. The association of Leydig cells with blood vessels is not obvious.

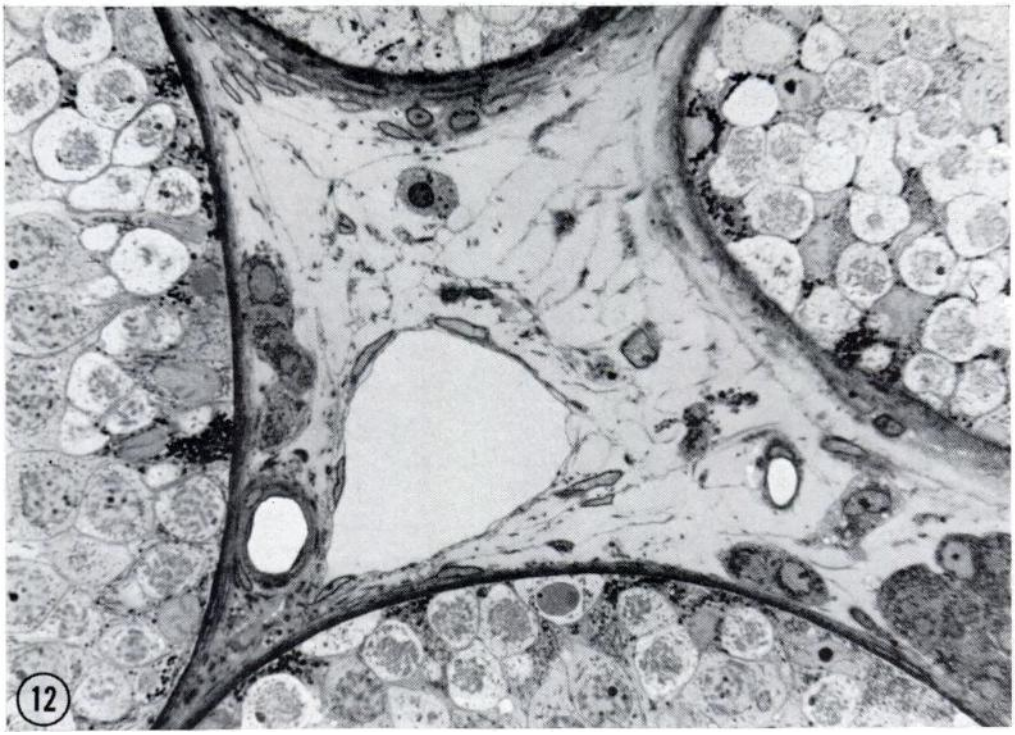
In discussing the tendency of Leydig cells in the human testicle to invade the lamina propria of the tubules and to invade loose areolar tissue around nerve sheaths in the spermatic cord and even to disrupt the nerve bundles, Halley (1960) suggested that this might be due to a paucity of available capillaries with which they might associate. We are unable to accept this interpretation, for in our specimens of the human testis, as in the monkey, intimate association of the Leydig cells with capillaries does not appear to be essential or even the rule. Moreover, there would seem to be an abundance of capillaries and by no means all of them have Leydig cells associated with their walls. Halley advanced the hypothesis that Leydig cells are situated between the arterioles and the seminiferous tubules and have the same capillary supply as the latter. The tubules would thus be downstream from endocrine cells and would therefore be first recipients of their androgens. Kormano and Suoranta (1971) seem to support Halley's thesis in emphasizing the close relation of circumferentially oriented capillaries to the lamina propria of the seminiferous tubules. Proximity of capillaries to the lamina propria may well be beneficial for other reasons, but the organization of the intertubular tissue in monkey and man suggests to us that androgen is released by Leydig cells directly into the intertubular tissue and that the appropriate concentration of androgen to sustain spermatogenesis is probably maintained in the abundant interstitial fluid and diffuses to and into the tubules around their entire circumference rather than being delivered via the blood to scattered sites where capillaries pass tangentially in the lamina propria of the tubules.

Ram (Ovis aries)

The topographical relationships of interstitial tissue components in the ram testis are not fundamentally different from those of primates. Leydig cells are relatively inconspicuous, occurring singly or in small clusters in an abundant connective tissue stroma that includes many fibroblasts and much collagen (Figs. 16, 17). The interstitium is well vascularized by blood vessels of large and small caliber. There is little evidence of preferential association of Leydig cells with the blood vessels. The overall density of the intertubular areas in stained sections and in electron micrographs (Fig. 18) is somewhat greater than in man. This is attributable to the greater abundance of collagen and to a high concentration of protein in the interstitial fluid. Many of the intertubular areas have a cen-



FIGS. 10 and 11. Photomicrographs of representative intertubular areas of Rhesus monkey testis. Leydig cells containing numerous lipid droplets occur singly or in clusters. They do not seem to be in any special relationship to blood vessels. Lymphatic vessels of varying size are a conspicuous feature of the interstitium in this species. Magnifications 701.3× and 765×.



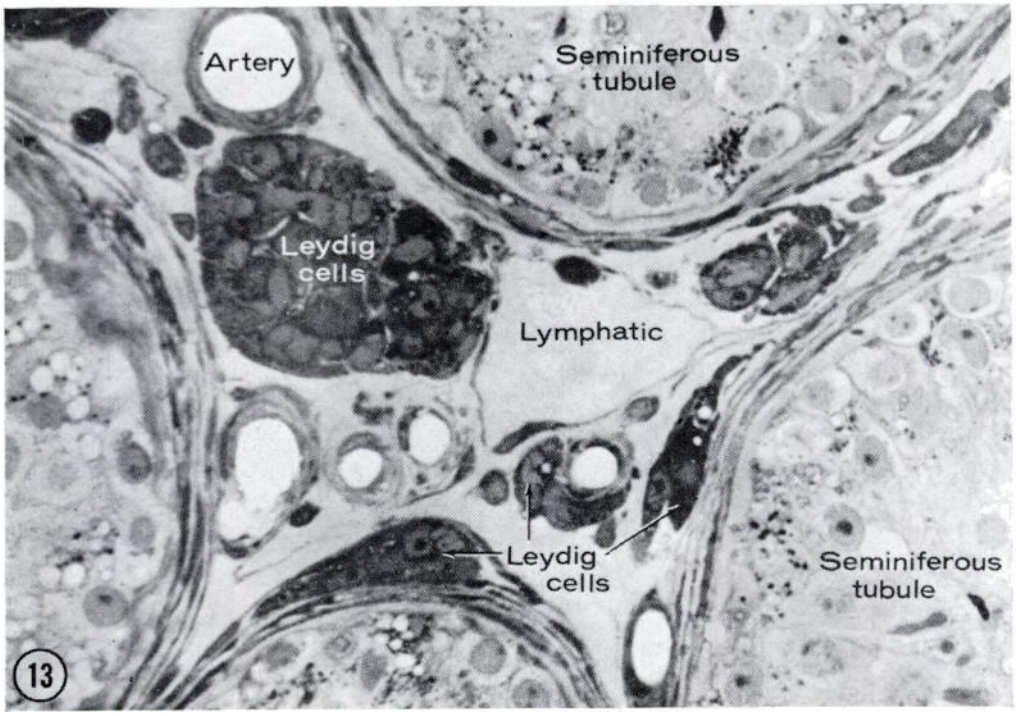
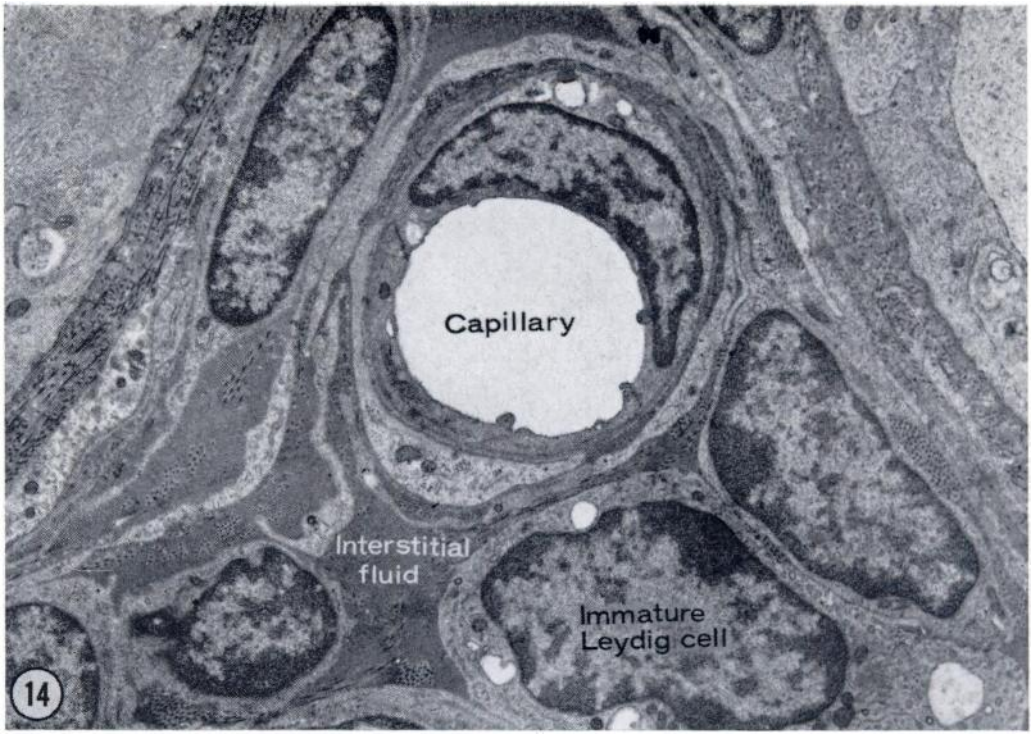
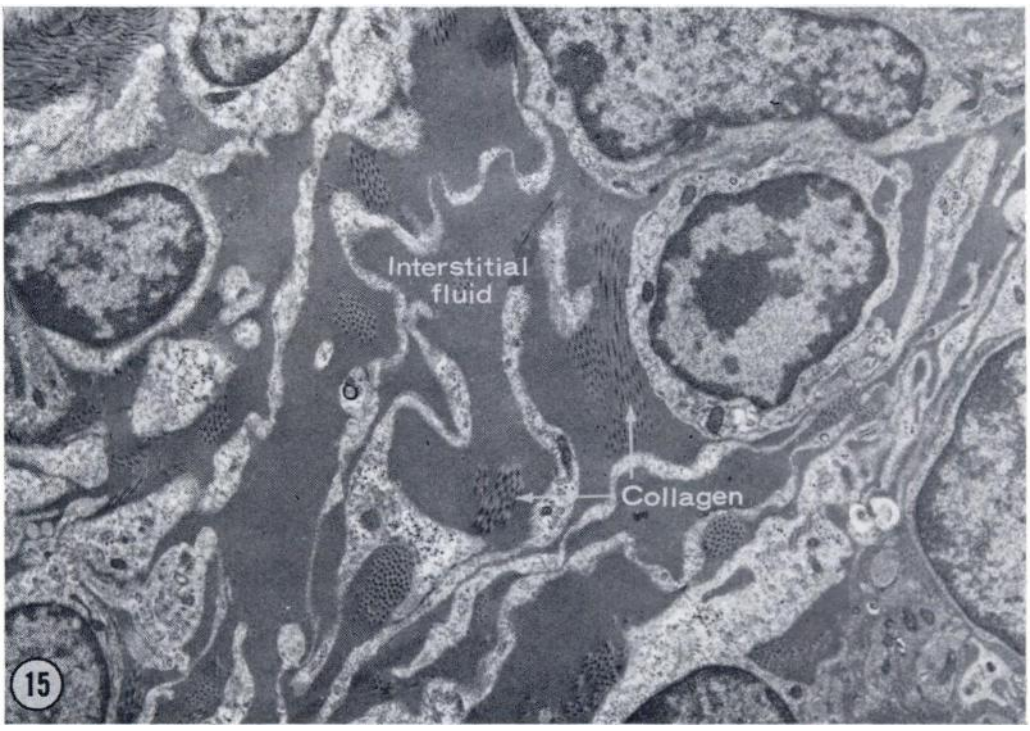


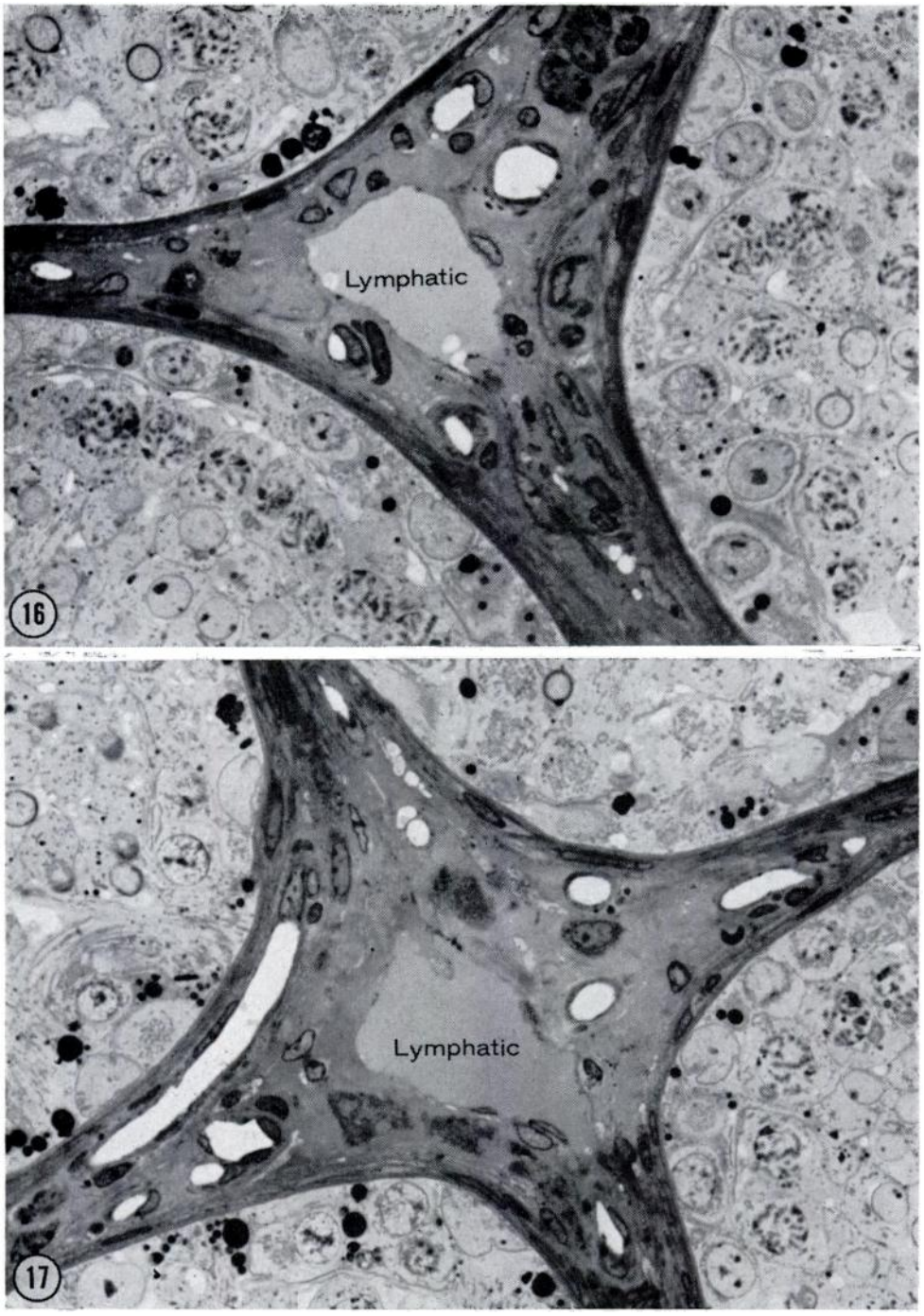
FIG. 12. Photomicrograph of an interstitial area of macaque testis. Notice the abundance of connective tissue space filled with a substance of appreciable density, and the large lymphatic vessel. Notice also that the Leydig cells are not very closely associated with the blood vessels. Magnification 495×.

FIG. 13. Photomicrograph of interstitial tissue from a perfused human testis. A large lymphatic is present and several clusters of Leydig cells. Only a few of the latter are perivascular. Magnification 450×. (Photograph courtesy of Dr. A. K. Christensen, from *The Human Testis* "Advances in Experimental Medicine and Biology," Vol. 10, Plenum Press, New York.)





FIGS. 14 and 15. Electron micrographs of interstitial tissue from an immature rhesus, infused for an hour with HCG, which induced a marked rise in circulating testosterone. Notice the surprising electron density of the fluid-filled interstitial spaces. This degree of density exceeds that of blood plasma. If it is due to the presence of plasma proteins, it would seem that these are more concentrated in the interstitial spaces than in the blood—and this seems unlikely. An alternative is to assume that some osmiophilic substance is present in the fluid in addition to plasma proteins. Magnifications 9000×.



FIGS. 16 and 17. Photomicrographs of two typical interstitial areas of the ram occupied by a vascular connective tissue with scattered clumps of Leydig cells. These are not very intimately associated with the small blood vessels. A large centrally placed lymphatic is quite characteristic of this species. Magnifications 510×.

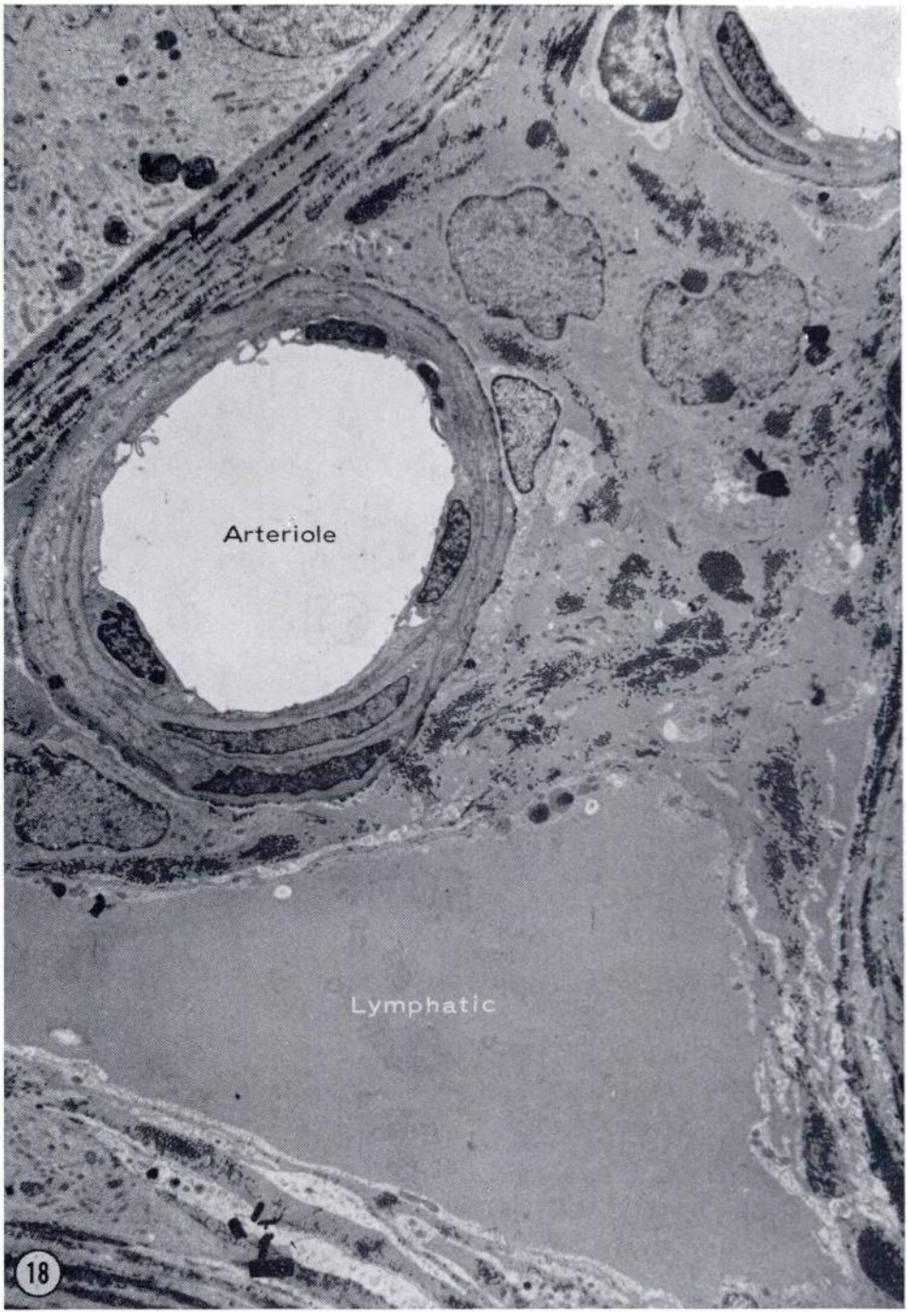


FIG. 18. Electron micrograph of ram testis. A lymph vessel is seen in the lower part of the figure. Connective tissue is denser than in rodent testis and in many other mammalian species. The ground substance of the connective tissue is of about the same density as the lymph. Magnification 2550×.

trally placed lymphatic vessel of fairly large size with a round or polygonal cross-sectional outline (Figs. 16–18). The endothelial lining of these lymphatics is continuous and supported by the dense collagen of the surrounding connective tissue.

Bull (Bos taurus)

The interstitial tissue of the bovine testis is basically similar in its organization to that of the ram but it is more difficult to study because of the abundance of collagen. The endocrine cells are in clusters of varying size that appear to be random in their distribution, some being perivascular, others unrelated to vessels and still others closely associated with the lamina propria of the tubules. Prominent lymphatic vessels are centrally placed in the intertubular areas. As a rule, there is one (Figs. 19 and 21) in each angular interspace but in some of the larger spaces, there may be two or three (Fig. 20). In material fixed by vascular perfusion, the lymphatics have a homogeneous grey content representing a precipitate of the protein of the lymph.

Coney (Heterohyrax brucei) *and Elephant* (Loxodonta africana)

The coney, an interesting small species, related to the elephant, is one of the few mammals that have internal testes. It is highly seasonal in its reproduction with active spermatogenesis for only a few weeks of the year. No morphological peculiarities of the testis have been observed that can be related to their tolerance of higher temperatures. The intertubular areas are largely occupied by an edematous areolar connective tissue permeated by blood vessels of large, as well as of small caliber. There is relatively little collagen compared to bull or ram. The Leydig cells comprise only a small fraction of the total volume of the interstitial tissue and occur in discrete clusters that seem to be preferentially associated with capillaries and venules (Figs. 23 and 24). Large irregularly shaped lymphatic vessels are of common occurrence and are highly variable in their size, position and cross-sectional outline. They may take up a greater fraction of a given intertubular area than is occupied by Leydig cells. (Fig. 24).

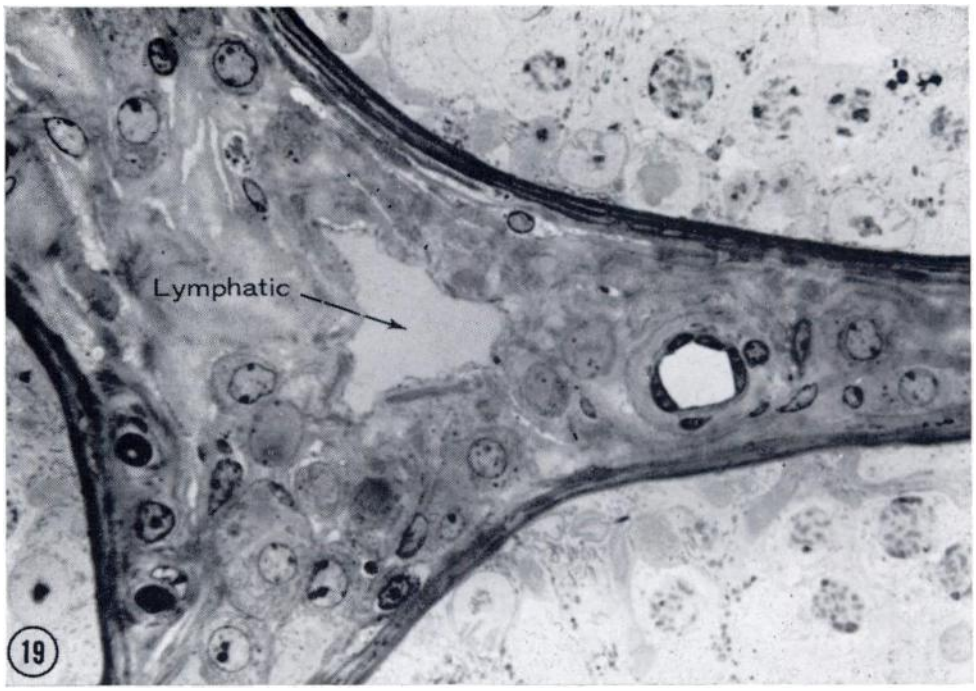
The interstitium of the African elephant testis is much like that of *Hyrax*. Compact clusters of lipid-rich Leydig cells are scattered in an abundant loose areolar tissue (Figs. 25 and 26). Collagen is not an especially prominent component. Lymphatics of large size are found in many of the intertubular spaces.

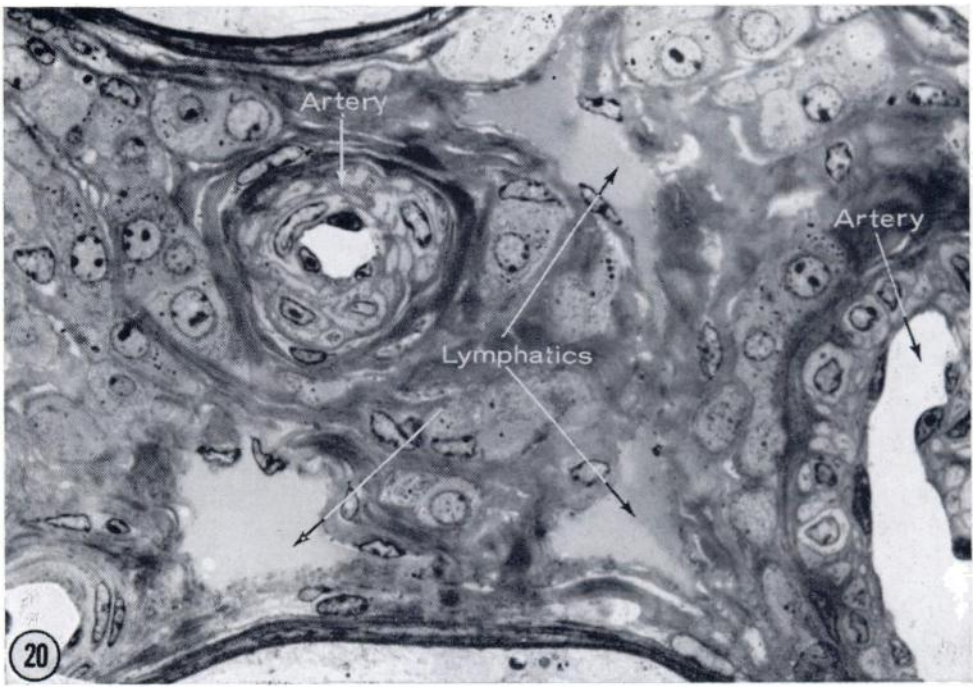
Opossum (Didelphys virginiana)

This North American marsupial has a large volume of highly cellular interstitial tissue. For the most part it consists of large, lipid-poor Leydig cells that stain quite darkly, but there are also many paler cells with a heterogeneous vacuolated cytoplasm that are interpreted as macrophages (Figs. 31 and 32). Scattered, relatively acellular areas are encountered, especially around blood vessels. These are occupied by a loose connective tissue stroma. As in other organs of the opossum, the blood vessels have uncommonly thick walls in relation to their caliber (Fig. 31). Lymphatic vessels are fairly abundant, often centrally located in the intertubular areas (Fig. 32). They are more numerous and of larger diameter than the lymphatics found in the other species that have an abundant and highly cellular interstitial tissue, such as the wart hog boar, domestic boar, and zebra described below.

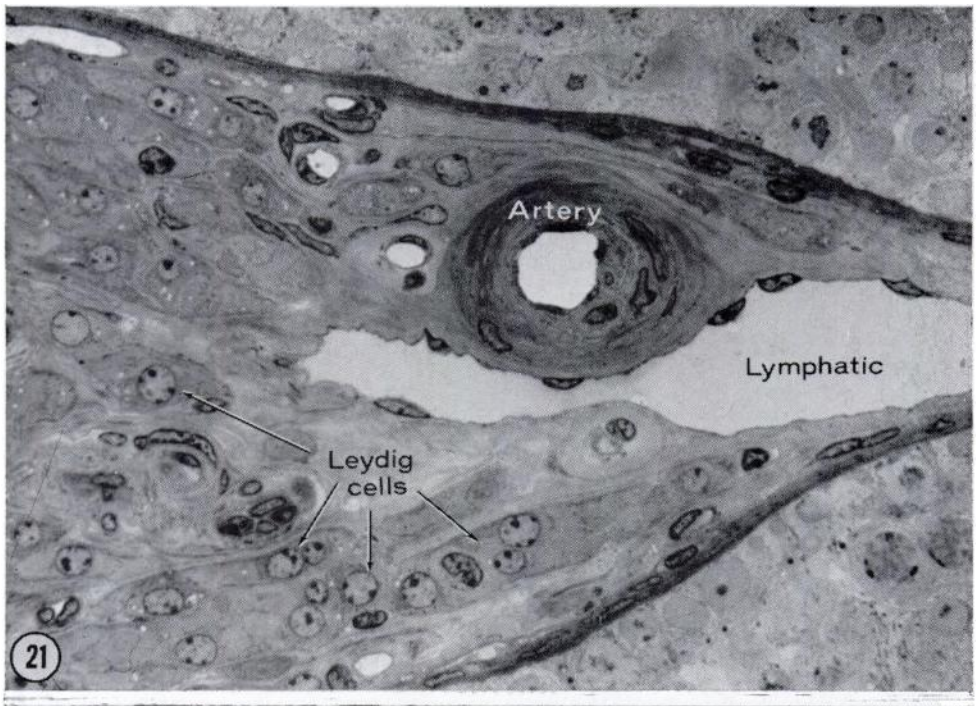
Domestic Boar (Sus scrofa), *and African Wart Hog* (Phacochoerus aetheopicus)

The testis of the boar is remarkable for the abundance of its interstitial tissue (Bascom and Osterud, 1927). The intertubular areas are conspicuously larger than in many other species and they are completely filled by large, polygonal Leydig cells so closely packed that they have the microscopic appearance of a solid epithe-





FIGS. 19 and 20. Photomicrographs of intertubular areas of bull testis. The Leydig cells are difficult to make out because of the relatively dense connective tissue. One or more lymphatic vessels of irregular outline are of common occurrence. Magnifications 637.5×.



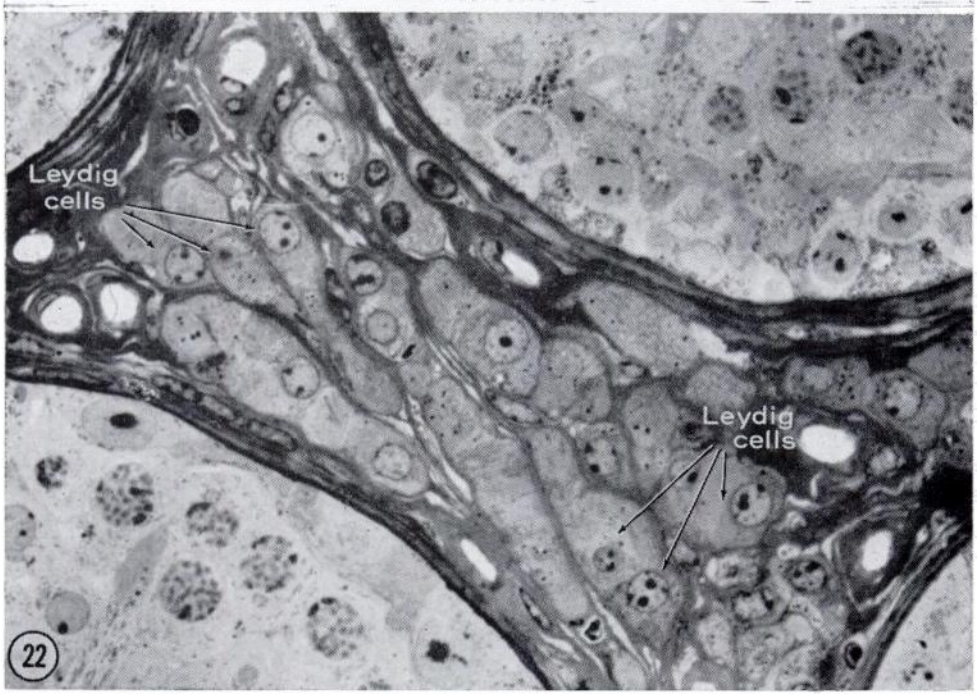
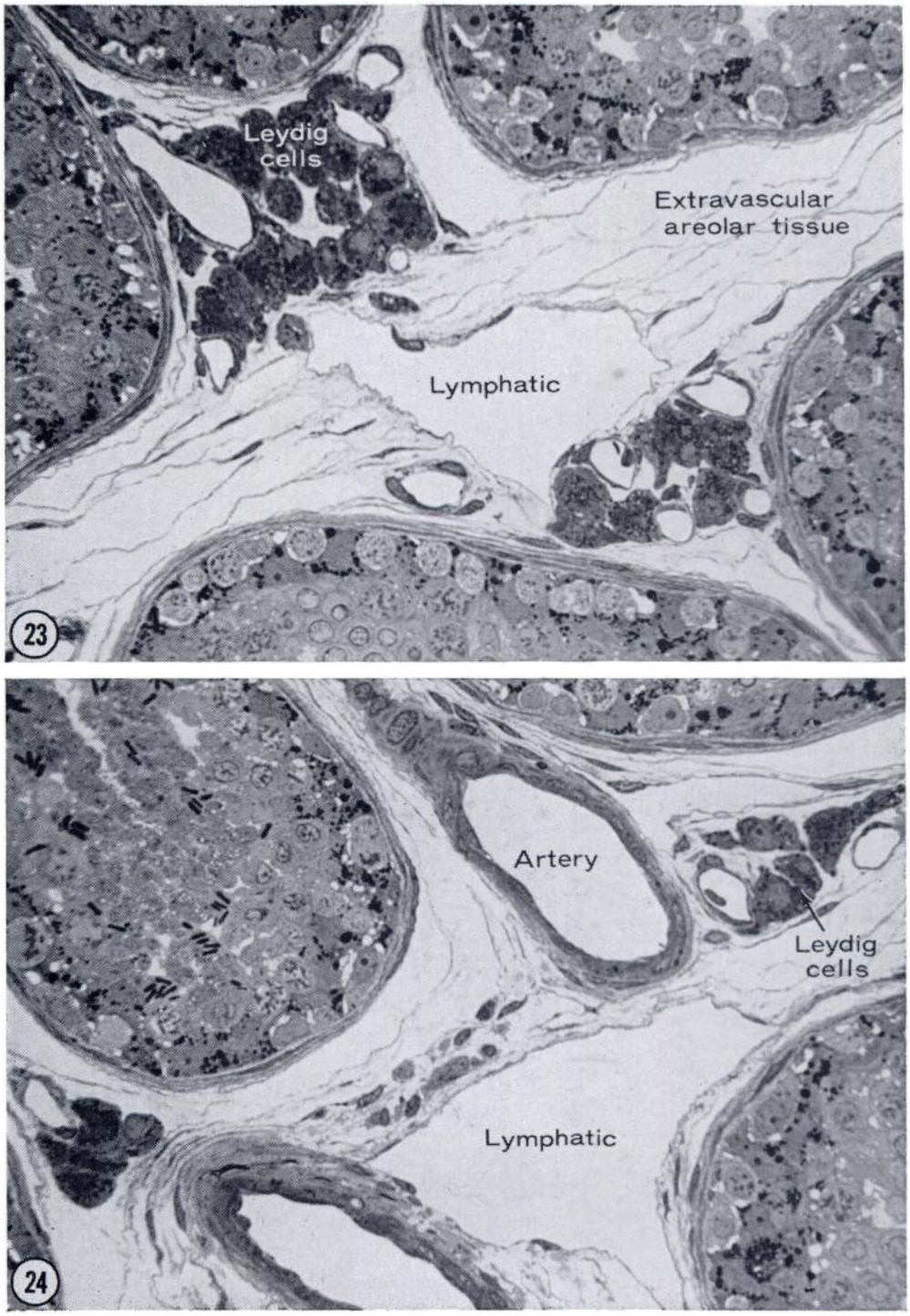
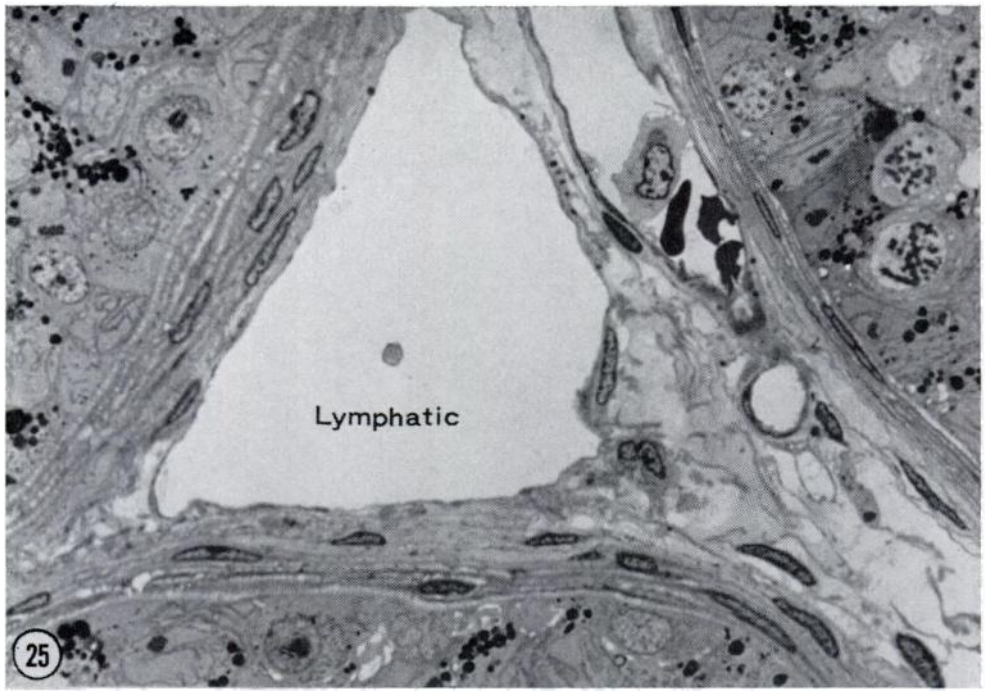


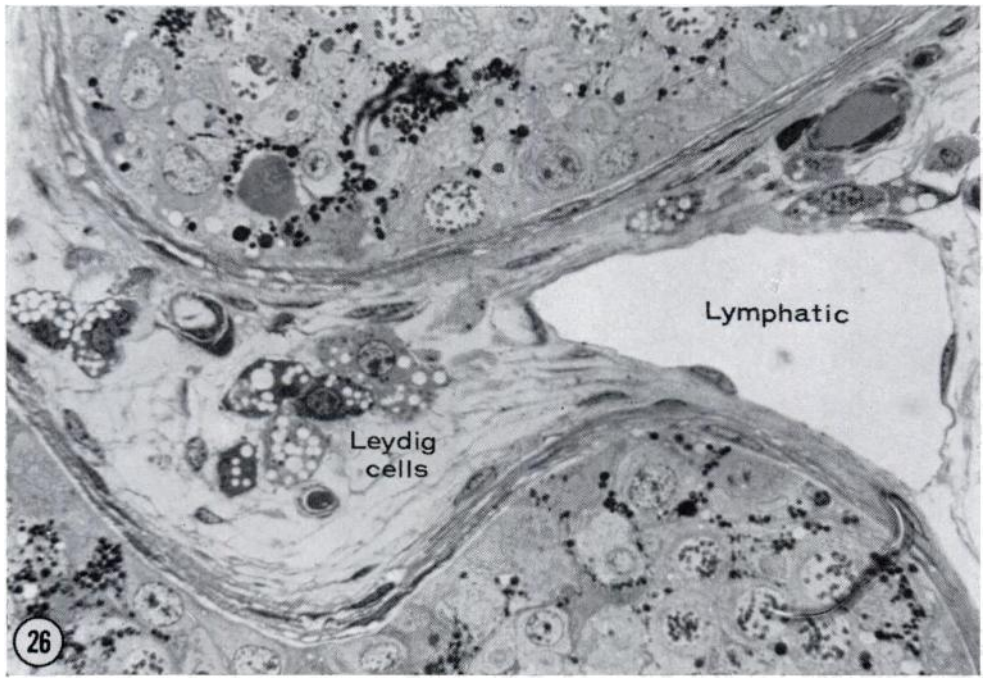
FIG. 21. Interstitial tissue of bull testis. An artery and a lymphatic are shown as well as numerous Leydig cells. Magnification 552.5×.

FIG. 22. An interstitial space of bull testis in which small blood vessels and large Leydig cells are embedded in a rather dense appearing connective tissue. No lymphatic vessel is present here. Magnification 680×.

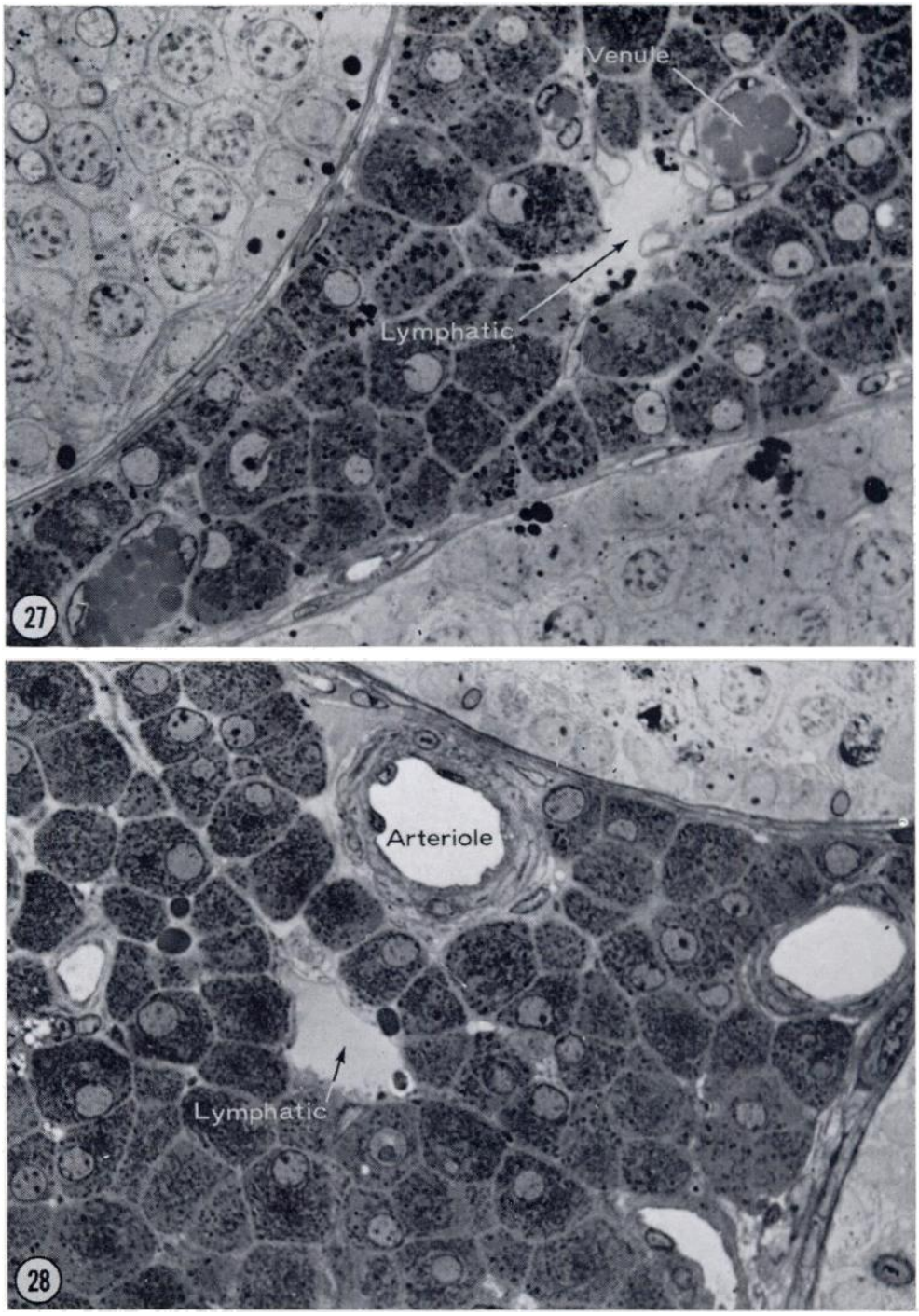


FIGS. 23 and 24. Photomicrographs of hyrax testis. Leydig cells are in clumps closely associated with small blood vessels. Loose areolar tissue occupies much of the intertubular area; large lymphatics are easily identified. Magnifications 405×.

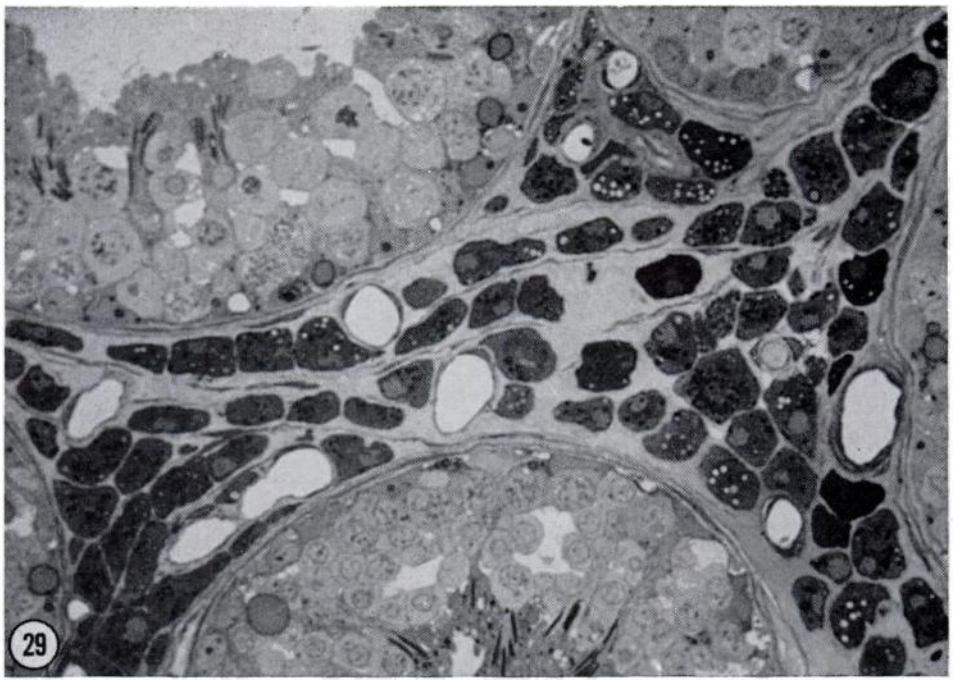


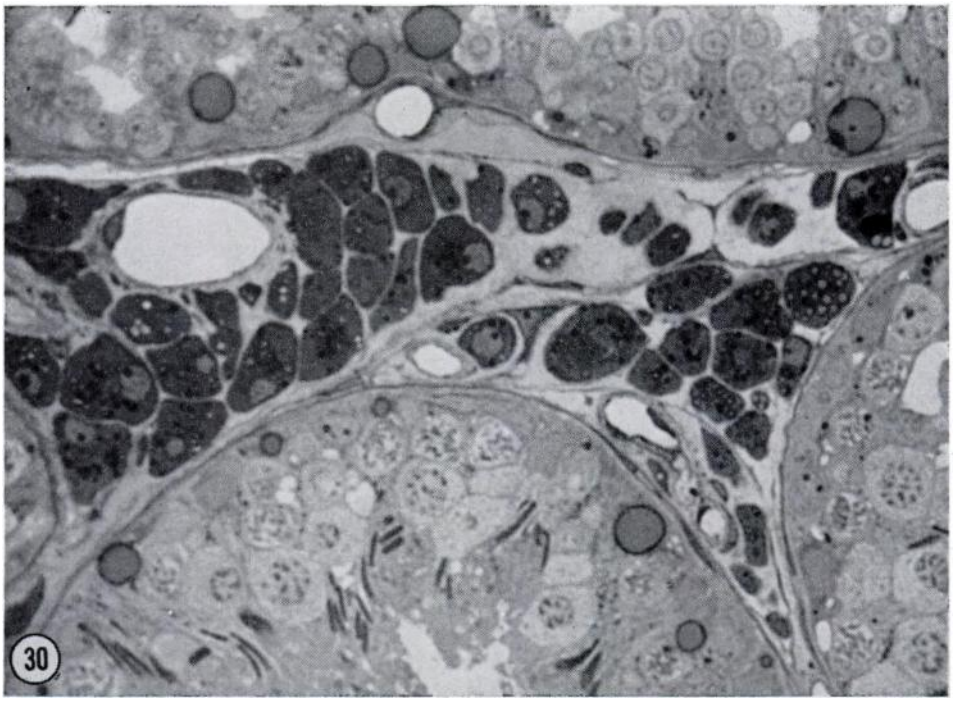


FIGS. 25 and 26. Photomicrographs of elephant interstitial tissue. The lipid-rich Leydig cells occur singly or in groups of varying size scattered in an abundant loose connective tissue. Lymphatics are numerous and of large size. Magnifications 680×.

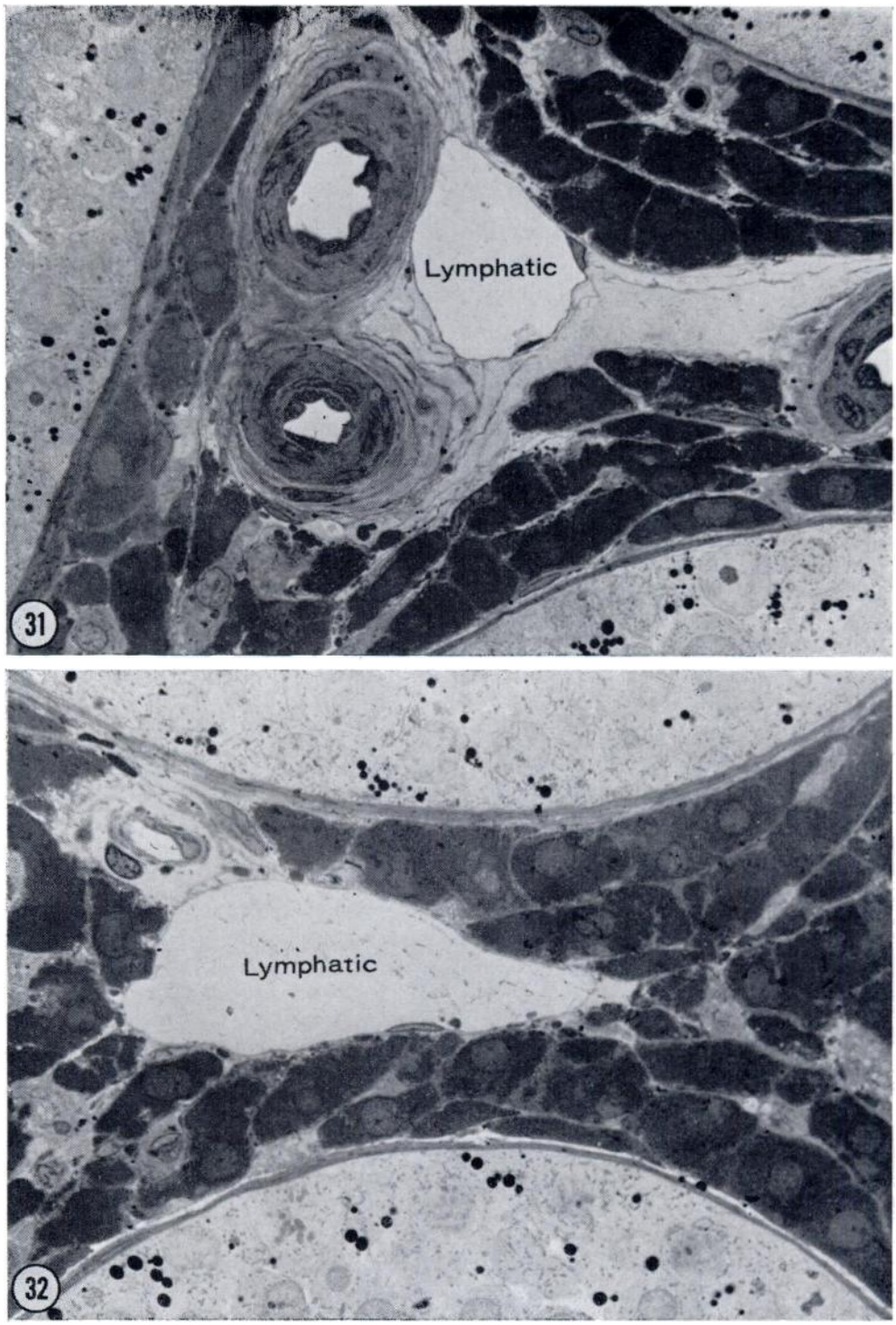


FIGS. 27 and 28. Two representative areas of interstitial tissue from domestic boar testis. There is a very large volume of closely packed Leydig cells and very little connective tissue. Blood vessels are numerous but interstitial lymphatics are very few and quite small. Magnifications 722.5×.





FIGS. 29 and 30. The wart hog, like the domestic boar, has a very large volume of Leydig cells. They are less densely packed, however, occurring as individual cells, rows, or clumps separated by the spindle-shaped cells of a very loose fluid-rich connective tissue. Lymphatics are rarely observed. Magnifications 467.5× and 595×.



FIGS. 31 and 32. Photomicrographs of interstitial areas from opossum testis fixed by perfusion. Large dense Leydig cells are very abundant but sizable centrally placed lymphatic vessels are of frequent occurrence. Magnifications 675×.

lial tissue. With the light microscope, the cells are lipid poor but very rich in mitochondria that tend to be concentrated in the cell center while the peripheral cytoplasm is relatively free of organelles other than smooth endoplasmic reticulum. The dense cellular mass is penetrated by numerous small blood vessels but very little perivascular or intercellular space is visible with the light microscope (Figs. 27 and 28). Despite their apparent crowding, the cells are found in electron micrographs to have numerous surface ruffles or microvilli that project into a system of narrow intercellular clefts 0.1–0.2 μm wide. These interspaces usually contain a dense material presumed to be protein. There is surprisingly little collagen associated with this highly cellular interstitial tissue. Interstitial lymphatics are very few and small. Many intertubular areas may have to be examined before locating a lymphatic (Fig. 28).

One might expect all members of the family Suidae to be quite similar to the domestic boar in the histology of their testis, but Parkes (1966) in a comparative study of suids has reported striking differences in the amount of interstitial tissue, the degree of "pigmentation," and in the occurrence of interstitial adipose cells. Significant differences have also been reported in the ultrastructure of the Leydig cells of the domestic boar and the African wart hog (Neaves *et al.*, 1974). In the wart hog, the interstitial tissue is very abundant and highly cellular as in the domestic boar, but there is considerably more extracellular space. The Leydig cells generally do not form as compact an epithelioid tissue as in the domestic boar. They are somewhat more loosely organized and often aligned in cords (Figs. 29 and 30). In some areas they may occur as individual cells separated by relatively wide intercellular spaces (Fig. 30). There is little collagen but fibroblasts with slender irregularly oriented processes are deployed between rows of Leydig cells or are randomly oriented in broad expanses of the interstitium that seem to be occupied only by the protein-rich matrix of a relatively acellular areolar tissue. Lymphatic vessels of small caliber are encountered, but only rather rarely.

Common Zebra (Equus burchelli)

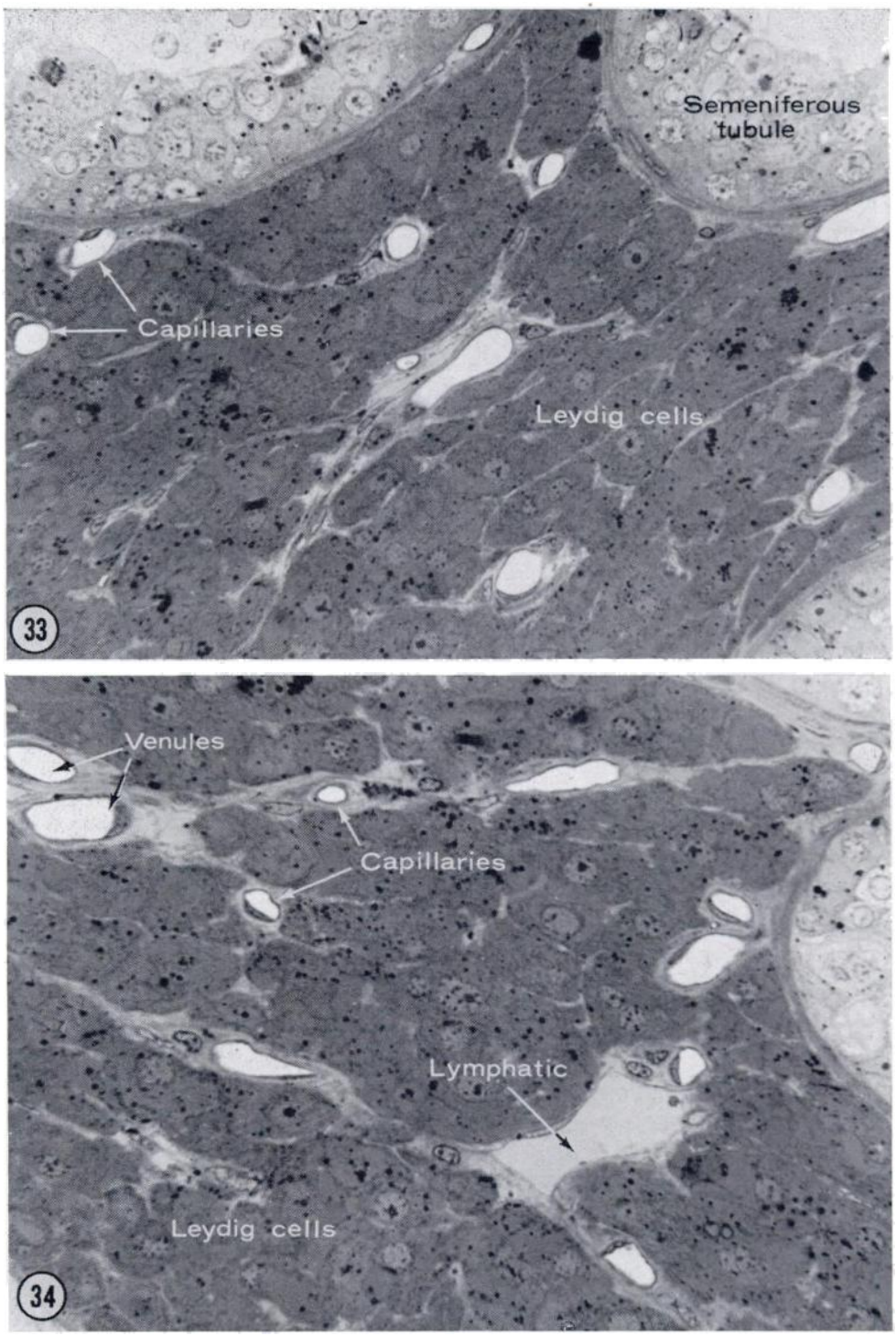
The equine testis is perhaps even more remarkable than boar testis in the proportion of interstitial tissue. In the common zebra, seminiferous tubules are separated by very large areas of endocrine tissue irrigated by a rich network of small blood vessels (Figs. 33 and 34). These course through a sparse connective tissue stroma that forms delicate septa traversing the masses of closely compacted Leydig cells. The interstitial lymphatics are poorly developed but occasional small lymph vessels are found in some of the larger intertubular areas (Fig. 34). These have no predictable location and may be either centrally placed or close to the tubules at the periphery of the intertubular areas.

On the basis of our observations on the boar, wart hog, and zebra, one is tempted to generalize that lymphatics are least well developed in those species that have the greatest volume of Leydig cells. The opossum, however, does not fit the generalization, but appears to be intermediate, having conspicuous lymphatics despite the unusual cellularity of its interstitial tissue.

Naked Mole Rat (Heterocephalus glaber)

This small, hairless, fossorial rodent is found in hot dry regions of Kenya, Abyssinia and Somalia. The four specimens that we were able to study were killed in Kenya in February and April. The seminiferous epithelium was rather inactive but did contain rare spermatocytes in prophase and a few late spermatids. It is not known whether spermatogenesis is seasonal, but the appearance of these specimens would suggest that it may be and that the animals studied here were in a period of declining spermatogenic activity.

The endocrine tissue of the testis was



FIGS. 33 and 34. The zebra is even more spectacular than the boar in the abundance of its Leydig cells. They form a dense epithelioid mass, subdivided by delicate connective tissue septa. There is a rich capillary bed, but lymphatics are rare. Magnification 495×.

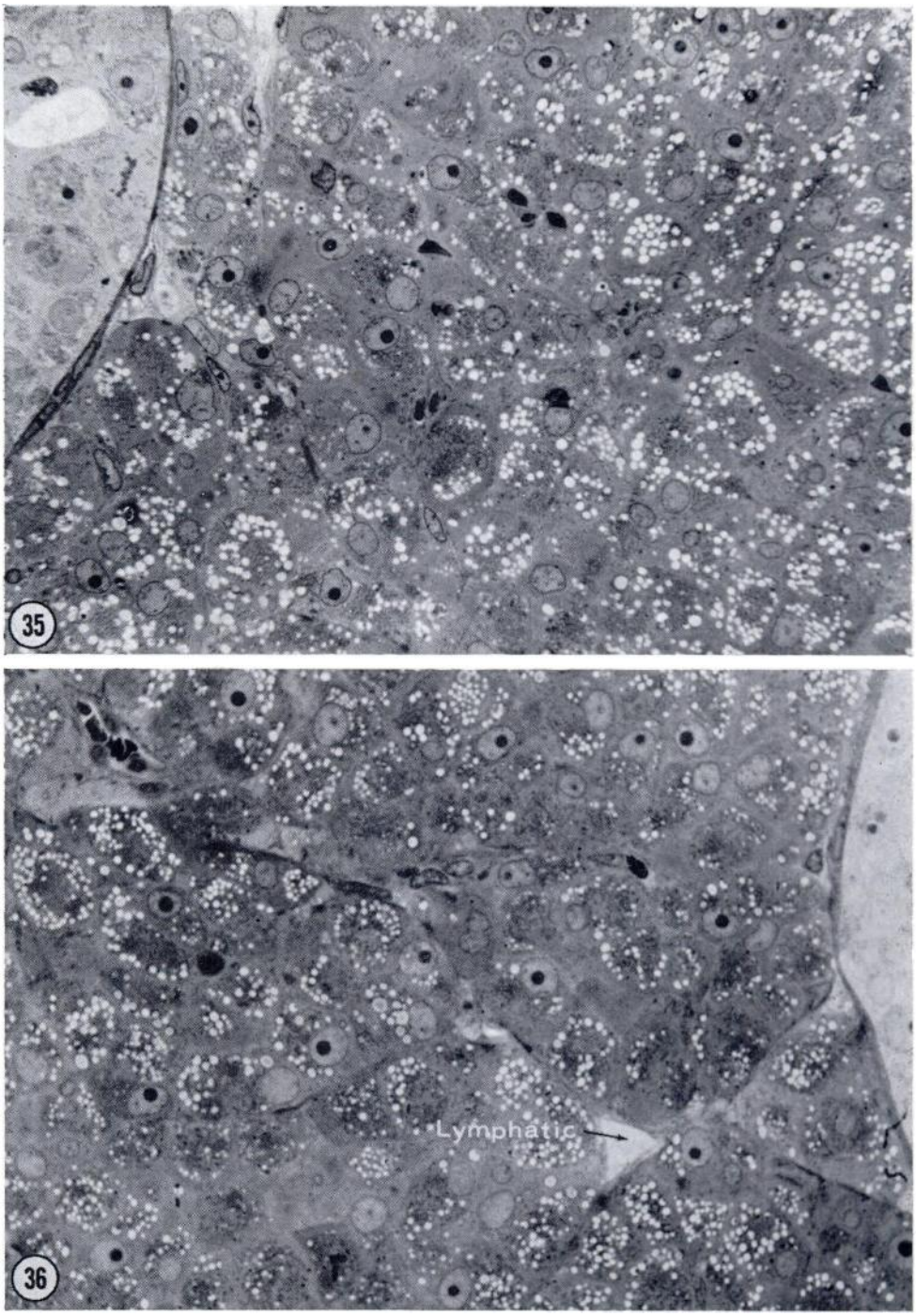
extraordinarily abundant, with seminiferous tubules sparsely distributed in a mass of lipid-rich Leydig cells (Fig. 35). The interstitial tissue was highly cellular, with little connective tissue stroma. Although lymphatics are no doubt present in small numbers, only one or two small vessels tentatively identified as such, were seen in rather careful light microscopic examination of sections from three different blocks (Fig. 36).

The findings in *Heterocephalus* with respect to volume of Leydig cells and degree of development of the interstitial lymphatics were in striking contrast to those on the common laboratory rodents. On the basis of an examination of a few laboratory species, one obviously cannot state that a small volume of Leydig cells and an extensive system of sinusoidal lymphatics is characteristic of rodents. It is especially surprising that the organization of the interstitial tissue in the testis of *Heterocephalus* is so very different from that of guinea pig and chinchilla, for in other aspects of its reproductive biology, particularly its long gestation and the presence of a vaginal closure membrane, this species would seem to have taxonomic affinities with Hystricomorph rodents (Jarvis, 1969).

DISCUSSION

A century of work on the histology of the testis produced no definitive answers to the questions: Do true lymph vessels or only lymph-filled interstitial spaces occur in the intertubular areas? Are there significant interspecific variations in the organization of the interstitium of the mammalian testis? If such differences exist, can they be explained in terms of phylogeny? Can they be correlated with specific variations in testicular physiology? Although our study leaves some of these questions unanswered, the widespread occurrence of interstitial lymphatics is no longer in doubt, and we have described interspecific differences in interstitial tissue organization that do not seem to follow phylogenetic lines. The physiological correlates of these variations in structural organization have yet to be determined.

The divergent views in the literature as to the presence or absence of interstitial lymphatics are attributable, in large measure, to the limited resolution of the light microscope and to the methods traditionally employed for demonstration of lymphatics. The results of the injection methods of the early investigators were discounted by later workers because of the potentially damaging injection media used, and the possibility of artifact due to unphysiological pressures. The later practice of studying stained histological sections after producing lymph stasis and edema by ligation of lymphatics in the spermatic cord was not without its own possibilities for artifact. This alternative to injection was also somewhat unphysiological and certainly capable of distorting normal relationships. Both of these classical approaches depended ultimately upon examination of histological sections with the light microscope. The cutting of tissue blocks from soft, fresh testis invariably causes considerable crushing and produces shearing forces between the firmer seminiferous tubules and the tenuous interstitial stroma that tend to disrupt structures as delicate as lymphatic vessels. To this mechanical damage is added the inevitable shrinkage or swelling involved in fixation, dehydration and embedding. And finally, in the 5–10 μm sections ordinarily viewed with the light microscope, it is exceedingly difficult to resolve the attenuated processes of slender fusiform cells well enough to determine whether they are endothelial cells bounding vessels or fibroblasts simply traversing edematous tissue spaces. Considering the multiple sources of artifact in traditional methods of specimen preparation and the inherent difficulties of interpretation, it is not surprising that the observations were in disagreement and the interpretations of doubtful validity. The improved preservation of the relationships of testi-



FIGS. 35 and 36. Photomicrographs of testis of the mole rat. The abundance of lipid-rich Leydig cells is quite spectacular. As in other species with highly cellular interstitial tissue, connective tissue is sparse and lymphatics are very few. Magnifications 510×.

cular components afforded by perfusion of aldehyde fixatives (Christensen, 1965; Fawcett *et al.*, 1969) combined with the added resolution achieved in light microscopy of plastic sections and by electron microscopy, have now made it possible to settle a number of issues in the persistent controversy over testicular lymphatics.

The variations in histology of the interstitium observed in fourteen species included in this study certainly cannot all be explained on phylogenetic grounds. Some fifty years ago, Lenninger (1923) and Stieve (1921), on the basis of rough estimates on nine or more species, reported that there may be great differences in the number and size of interstitial cells within the same family. Parkes (1966) has recently described wide variations in relative amount and distribution of interstitial tissue in six species of the subfamily Suinae. The bearded pig, *Sus barbatus*, for example, has a relatively modest amount while the forest hog, *Hylochoerus meinertzhageni*, and the African wart hog, *Phacochoerus aethipicus*, have very large amounts. In this paper we add the zebra, *Equus burchelli*, the opossum, *Didelphys virginiana*, and the mole rat, *Heterocephalus glaber*, to the list of species with unusually abundant, highly cellular testicular interstitial tissue. We also draw attention to a striking example of extreme variation within the same order. The interstitial cells of guinea pig, *Cavia porcella*, represent approximately 2% of the volume of the testis whereas in the fossorial rodent, *Heterocephalus glaber*, these cells are estimated to comprise over 60% of the testicular mass. Taylor and Horner (1970) have reported extreme variation in the abundance of interstitial tissue within a single genus of Australian murid rodents. *Leggadina hermannsburgensis* shows the small proportion of interstitial tissue usual among murid rodents while *Leggadina delicatula* possesses an extreme abundance of Leydig cells.

Why some species have very little endocrine tissue in the testis while others have a very large amount remains unexplained and to our knowledge without a parallel in interspecific variations in other endocrine glands. The domestic boar and the stallion have in common the secretion of large amounts of estrogen as well as of androgen. The urinary excretion of estrogen is high in both and in both there is an unusual abundance of Leydig cells. The biological significance of high rates of estrogen secretion in the males of these species is unexplained. It would now be of interest to study estrogen levels of *Heterocephalus*, and of *Didelphys* to see how well this characteristic correlates with relative abundance of Leydig cells. Moreover, as Parkes (1966) has pointed out, the testis of the domestic boar is to date the only known source of the odorous steroid Δ^{16}-androstenon which is thought to serve as a pheromone (Sink, 1967). Considering the abundance of Leydig cells in the boar testis and their possible involvement in synthesis of a steroid other than testosterone, it is significant that Claus *et al.* (1971) have reported ten times as much 5α-androst-16-en-3-one as testosterone in boar testis. It is conceivable that in some other species, the unusual abundance of interstitial tissue may be related to synthesis of steroid products other than testosterone—pheromones or substances with some other functions as yet undefined.

Fossorial rodents and insectivores seem to have in common a large volume of testicular endocrine tissue. The woodchuck (*Marmota monax*) is well known for the quantity of its interstitial tissue (Rasmussen 1917: Christian *et al.*, 1972). The European mole (*Talpa europea*) also is said to have voluminous interstitial tissue consisting of very active appearing Leydig cells (Folliot and Picheral, 1972). To these we have added the naked mole rat (*Heterocephalus glaber*). Until more is known about the endocrinology of these animals, one can only speculate that olfactory signals would be more useful to fos-

sorial species in the Stygian blackness of the burrow than behavioral signs of estrus or rut which would require visual perception.

The present study records observations on representative species of rodents, artiodactyls, perissodactyls, marsupials, hyracoids, probosideans, and primates. A broad range of variation was found in the relative proportions of the principal components of the interstitial tissue, but if one compares species with respect to volume of Leydig cells, volume of interstitial connective tissue, and the location and degree of development of parenchymal lymphatics, it is possible to distinguish three general patterns of organization:

In the first category are those species which have a relatively small volume of Leydig cells (1–5% of the volume of the testis) and a minimum of interstitial connective tissue. The greater part of the intertubular area in these animals is occupied by extensive peritubular lymphatic sinusoids with continuous or discontinuous endothelial lining. This category includes guinea pig, chinchilla, rat, and mouse.

In the second category are those species in which clusters of Leydig cells are widely scattered in a very loose connective tissue stroma drained by conspicuous lymphatic vessels, more or less centrally located in the interspaces. To this category belong ram, bull, hyrax, elephant, monkey, and man.

In the third category, closely packed epithelioid Leydig cells are the dominant component of the interstitium (20–60% of the testis volume). They fill nearly all of the intertubular area. There is very little interstitial areolar tissue and lymphatics are very few and of small caliber. To this group belong the domestic boar, wart hog boar, zebra, opossum, and naked mole rat.

Although the functional implications of the different patterns of interstitial tissue organization cannot be specified at present, a few speculations can be offered. The testis is unique among endocrine glands in *not* having fenestrated capillary endothelium (Wolf and Merker, 1966; Fawcett *et al.*, 1970). Nevertheless, a common feature of the intertubular tissue of all the species studied was the presence of a fine-grained precipitate of plasma protein throughout the extracellular compartment. This precipitate is interpreted as a morphological expression of an unusual permeability of interstitial capillaries and venules to plasma proteins. The high degree permeability of these vessels has been clearly demonstrated with isotopic, fluorescent, and enzymatic tracers (Everett and Simmons, 1958; Mancini *et al.*, 1965; Dym, 1973). The need for unusual permeability of the interstitial vascular bed is not immediately obvious; but it is obvious from our studies that the interstitium of the testis, in many of the species, normally contains an amount of fluid that would be described as *edema* if observed in the connective tissue of other organs. A copious outpouring of protein-rich fluid requires a sink of corresponding capacity. The interstitial lymphatics serve to drain this abundant extracellular fluid and extravasated plasma protein back to the general circulation. Future physiological measurements of vascular permeability, in the several species studied here, may well reveal differences comparable to the specific differences we have found in the degree of development of their lymphatics.

In the category exemplified by the guinea pig where the Leydig cells are clustered around centrally located vascular elements, the protein-rich fluid escaping from the blood capillaries traverses the visceral endothelium and fills lymphatic sinusoids that are everywhere interposed between the interstitial cells and the tubules. Similarly in the rat and mouse, but in these, the visceral layer of endothelium is largely lacking so that the Leydig cells are actually within a lymphatic sinusoid and are directly exposed to the lymph. In these laboratory rodents the blood vessels are

not immediately adjacent to the tubules. The peritubular lymphatic sinusoids or lymph spaces must therefore play an important role in access of nutrients and androgens to the seminiferous epithelium. Although the volume of endocrine tissue is small, the Leydig cells are strategically located in the path of a continuous slow movement of protein-rich extracellular fluid from the blood to the lymphatic sinusoids. The tubules are downstream from the Leydig cells in this flow. An inevitable consequence of this arrangement would seem to be that the androgen secreted by the Leydig cells is partitioned between the venous blood and the lymph. Owing to the more rapid rate of blood flow, the total amount of androgen reaching the general circulation via the blood vascular system no doubt greatly exceeds that leaving the testis via the lymphatics. On the other hand, due to the brief sojourn of androgen of the venous blood in the testis and its rapid dilution in the circulation, this androgen would seem to be less important for the spermatogenic function of the testis than the androgen concentration that is maintained in the peritubular lymphatics. It seems likely that a considerably greater concentration of testosterone may build up in the slow flowing lymph in the peritubular sinusoids than would be found in the more voluminous and rapidly circulating blood. This differential may well be physiologically significant.

In the category exemplified by ram or monkey, the Leydig cell clusters are scattered in a loose areolar stroma which is drained by centrally placed lymphatic vessels. The blood vessels are often peripheral and may be firmly attached to the lamina propria of the tubules. In these species, the tubules cannot be easily teased away from the interstitial tissue (Christensen and Mason, 1965). Androgen and nutrients evidently diffuse to the tubules through the fluid phase of the edematous ground substance. The lymphatic vessels are probably mainly concerned with return of protein to the general circulation. The turnover of the fluid phase of the loose areolar tissue in these species may well be slow relative to the emptying time of the peritubular lymphatics of rodents. In these species, therefore, it may be possible to maintain relatively higher local concentrations of androgen than in the guinea pig or rat.

In the category exemplified by the boar, blood capillaries are uniformly distributed through the compact epithelioid interstitial tissue. In the absence of a significant amount of connective tissue, the extracellular fluid occupying the interstices among Leydig cells would seem to be less abundant than in species of the first and second categories. In view of the relatively small extracellular fluid volume in this group, it is perhaps not surprising that the lymphatics are few, small and widely scattered. Whether the large mass of Leydig cells provides high local concentration of androgen despite a seemingly inefficient delivery system remains to be determined.

It is known that testicular lymph of the ram contains appreciable amounts of testosterone (Lindner, 1963). In view of the origin of the lymph and its high protein content, it is natural to think of the possible role of specific carriers. Hormone binding globulins have been identified or postulated for thyroxin, insulin and several steroids (De Moor *et al.*, 1968). Transcortin, an α-glycoprotein, preferentially binds adrenal glucocorticoids but also binds testosterone and 1-β-estradiol. A steroidbinding β globulin has also been described which has a high affinity for testosterone (Pearlman and Crepy, 1967; Vermuelen and Verdoncle, 1968). The physiological importance of these factors has yet to be assessed. One can speculate, however, that if an androgen-binding protein of the plasma were involved in transport of testosterone to distant target organs, a high permeability of the intertubular vessels would insure continuous access of carrier protein to the surface of the Leydig cells.

While such a mechanism might be beneficial for transport of hormone back to the general circulation, it would clearly be of little value in the primary function of androgen in sustaining spermatogenesis, for binding to a protein carrier would result in its exclusion by the permeability barrier that resides in the wall of the tubules (Setchell *et al.*, 1969; Dym and Fawcett, 1970). It seems likely that free testosterone is required for this local function if, as we assume, penetration of androgen into the epithelium is required.

Some of speculations advanced here may well prove to be erroneous, but it seems inescapable to us that the strikingly different patterns of organization in the interstitial tissue of the mammalian testis must be reflected in specific differences in the nutrition of the seminiferous epithelium and its fluid secretion; in the partition of androgens between blood and lymph; in the rate of lymph flow; and in differences in the efficiency with which a critical concentration of androgen is maintained around the tubules. Until now, variations in the morphology of the interstitium have been largely ignored. It is hoped that this paper will stimulate others to take these differences into account in future physiological studies of the testis.

ACKNOWLEDGMENTS

It is a pleasure to acknowledge the generosity of Dr. Jennifer Jarvis of the Zoology Department, University College, Nairobi for the specimen of *Heterocephalus glaber* Ruppell.

We are indebted to Dr. R. P. Amann of the Department of Animal Science, Pennsylvania State University for the opportunity to fix a bull testis by perfusion. Our thanks to Dr. Kent Christensen who has kindly permitted us to reproduce one of his micrographs of perfused human testis and to Dr. Winston Anderson and Dr. Vitale-Calpe who have supplemented our own material on rat and mouse.

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