MORPHOMETRIC ANALYSIS OF LEYDIG CELLS IN THE NORMAL RAT TESTIS

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ABSTRACT

Leydig cells are thought to be the source of most, if not all, the testosterone produced by the testis. The goal of this study was to obtain quantitative information about rat Leydig cells and their organelles that might be correlated with pertinent physiological and biochemical data available either now or in the future. Morphometric analysis of Leydig cells in mature normal rats was carried out on tissue fixed by perfusion with buffered glutaraldehyde, and embedded in glycol methacrylate for light microscopy and in Epon for electron microscopy.

In a whole testis, 82.4% of the volume was occupied by seminiferous tubules, 15.7% by the interstitial tissue, and 1.9% by the capsule. Leydig cells constituted 2.7% of testicular volume. Each cubic centimeter (~1 g) of rat testis contained about 22 million Leydig cells. An average Leydig cell had a volume of 1,210 μm^3 and its plasma membrane had a surface area of 1,520 μm^2.

The smooth endoplasmic reticulum (SER), the most prominent organelle in Leydig cells and a major site of steroidogenic enzymes, had a surface area of ~10,500 μm^2/cell, which is 6.9 times that of the plasma membrane and is 60% of the total membrane area of the cell. The total surface area of Leydig SER per cubic centimeter of testis tissue is ~2,300 cm^2 or 0.23 m^2. There were 3.0 mg of Leydig mitochondria in 1 g of testis tissue. The average Leydig cell contained ~622 mitochondria, measuring on the average 0.35 μm in diameter and 2.40 μm in length. The mitochondrial inner membrane (including cristae), another important site of steroidogenic enzymes, had a surface area of 2,920 μm^2/cell, which is 1.9 times that of the plasma membrane. There were 644 cm^2 of inner mitochondrial membrane/cm^3 of testis tissue.

These morphometric results can be correlated with published data on the rate of testosterone secretion to show that an average Leydig cell secretes ~0.44 pg of testosterone/d or 10,600 molecules of testosterone/s. The rate of testosterone production by each square centimeter of SER is 4.2 ng/d or 101 million molecules/s; the corresponding rate for each square centimeter of mitochondrial inner membrane is 15 ng testosterone/d or 362 million molecules/s.

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Testicular Leydig cells are the principal source of androgens in the male. The fine structure of these cells and the localization of steroidogenic enzymes within their cytoplasm has been described in reviews (8, 9). The most prominent ultrastructural features exhibited by these cells are an abundant smooth endoplasmic reticulum (SER) and fairly numerous mitochondria. Lipid droplets are common in Leydig cells of some species, although not in those of adult laboratory rats. The biosynthesis of testosterone is catalyzed by enzymes located predominantly on membranes of the SER and in adjacent cytoplasm, although a few steps occur on the inner mitochondrial membranes. The total Leydig cell population in 1 g of rat testis produces 6.7 ng of testosterone/min in vivo (14). If we could determined the number of Leydig cells in that amount of testis, it would be possible to calculate the rate of testosterone production per average Leydig cell. Furthermore, if the surface area of intracellular membranes on which steroidogenic enzymes occur could be measured, then the average rate of testosterone synthesis per unit area of membrane could be determined, furnishing some insight into biochemical efficiency at the membrane level. Other quantitative information about structures within the cell may allow functional correlations in the future, as more detailed biochemical information becomes available.

The use of quantitative methods at the light and electron microscope levels has increased remarkably over the last few years. These methods (5, 12, 28), collectively called "morphometry" or "stereology," allow the number, volume, surface area, and lineal extent of structures in three dimensions to be assessed from the two-dimensional images of light and electron microscopy. Several papers have appeared on various aspects of testis morphometry (see Discussion), but a broad morphometric study of normal Leydig cells has not been available.

In another study from this laboratory, Christensen and Peacock (10) have used morphometry on histological sections at the light microscope level to follow changes in the number of Leydig cells in adult rats under chronic treatment with human chorionic gonadotropin (hCG). They found that control testes contained an average of ~18.6 million Leydig cells/cm^3 tissue. When the rate of testosterone production by testes in vivo (14) was divided by this number, the result suggested that an average individual Leydig cell secreted ~0.5 pg of testosterone/d, or 12,500 molecules/s.

The present study includes an independent estimate of Leydig cell number in rat testes prepared for light microscopy by a recent method that provides superior preservation (2). In addition, morphometric analysis is also carried out at the electron microscope level, allowing quantitative information about Leydig cell organelles to be correlated with data on the rate of testosterone synthesis.

MATERIALS AND METHODS

Tissue Preparation

Four adult male rats (Sprague-Dawley, from West Jersey Biological Supply, Wenonah, N. J.), weighing 255, 265, 345, and 365 g, were used after being maintained ad lib. on normal lab chow. 2 d before sacrifice, animals were injected intraperitoneally with 3% trypan blue (1 ml/100 g body weight) to label macrophages in the interstitial space, because these cells are numerous in the rat testis and might be mistaken for Leydig cells (8, 9). Even though the trypan blue is extracted from the cells during later preparation for light or electron microscopy, the abundant empty lysosomes give the macrophages a distinctive pale appearance, allowing them to be distinguished easily from Leydig cells.

After an animal was placed under ether anesthesia, both testes were fixed by perfusion (7) with 3% glutaraldehyde buffered with 0.1 M *s*-collidine, (pH 7.4, 438 mosmol) for 30–60 min at room temperature. Several other fixatives were tried, in which the concentrations of glutaraldehyde and *s*-collidine were varied, or other buffers were utilized, but the above fixative gave the best morphology for rat testes at both light and electron microscope levels. After perfusion the testes were cut into ten slices, perpendicular to the long axis of the testis, and were washed in buffer overnight or longer. Alternating slices were utilized for light and electron microscopy, making five slices for each.

Slices for light microscopy were dehydrated in ethanol (20–30 min each in 50, 70, 80, 90, 95 and 100%), and were embedded in glycol methacrylate (GMA) (JB-4 embedding medium, Polysciences Inc., Warrington, Pa.), according to the method of Bennett et al. (2). The slices, oriented to provide full testis cross sections, were sectioned at a setting of 2 μm with a JB-4 microtome (Dupont Instruments-Sorvall, DuPont Co., Newton, Conn.) and dry sectioning on a glass knife (2). Sections were subjected to double staining with Weigert's iron hematoxylin (20) and with toluidine blue-basic fuchsin, modified from Lee's methylene blue-basic fuchsin method described by Bennett et al. (2). This double staining allows the Leydig cells to be easily recognized and clearly distinguished from macrophages.

The remaining five slices were cut into small pieces for electron microscopy. Two to four pieces from each slice were postfixed with unbuffered 2% osmium tetroxide and 1.5% ferrocyanide (17), dehydrated with graded ethanols, and embedded in Epon 812. Thin sections of silver to pale gold interference color were cut with a Porter-Blum MT-2B ultramicrotome (DuPont Instruments-Sorvall, DuPont Co.) with a diamond knife, and were stained for 10–20 min with saturated or 2% aqueous uranyl acetate and for 5–10 min with lead citrate (25). Micrographs were taken on a JEOL 100B electron microscope at 80 kV. Magnifi-

cations were determined by means of a carbon grating replica.

Although both testes were prepared from each animal, only one was used in the study, the choice being made after the quality of preservation was established by preliminary examination by light and electron microscopy.

Morphometric Procedures

In this study we utilized current methods and formulae of morphometry or stereology, as described in the valuable reviews of Weibel and Bolender (28) and of Elias et al. (12), to which the reader is referred for background and derivations. All calculations were carried out with a Hewlett-Packard HP-27 hand calculator (Hewlett-Packard Co., Palo Alto, Calif.).

LIGHT MICROSCOPY: Sections for light microscopy were viewed with a Zeiss research microscope fitted with Neofluar objective lenses. To estimate the volume density (V_V) of seminiferous tubules and interstitial tissue, five sections per animal were studied with a × 6.3 objective and an eyepiece grid containing a square lattice of 441 points. The number of hits on pertinent structures over the whole surface of each section was counted by moving the section in steps back and forth across the grid, being careful that structures under one edge of the grid were placed just beyond the other edge for the next step, thus leaving no area uncounted. Volume density was obtained by dividing the sum of the points falling on each structure by the total test points lying over the tissue. The volume of the testicular capsule was estimated by multiplying the mean width of the capsule by the testicular surface area, as determined geometrically from the long and short axes of the testis.

Counts used to determine the volume and numerical densities of Leydig cells were made with a × 40 objective lens and the same eyepiece grid. A total of 300–400 grid fields were counted on each specimen, the position of each field being determined blindly by bringing the stage to successive units of its calibrated scale. The volume density of the cells was derived from the number of hits on either the nucleus or cytoplasm of Leydig cells, compared to the total test points lying over the tissue. A similar approach was used to measure the volume density of Leydig nuclei.

The numerical density (N_V), or number of Leydig cells per unit volume of testis, was calculated from counts of the number of Leydig cell nuclei per unit area of section (N_A), from measurements of the average nuclear diameter (D), and from the average thickness of the sections (T), utilizing the Floderus equation (13):

$$N_V = N_A/(T + D - 2h),$$

where h is the height of the smallest recognizable cap or grazing section of the nucleus. The counts for N_A were made at the same time as the volume measurements described above, and consisted of counting the number of Leydig cell nuclei included within the perimeter of the successive grid fields (each with a surface area of 0.1 mm^2 on the section). Nuclear profiles overlapping the boundary of the field were counted if they were on the upper or left boundary, but were ignored on the lower and right boundary. The mean diameter of the nucleus D was obtained by direct measurements of its long and short axes, using sections 10 μm thick; nuclei were counted only if the optical middle of the nucleus, where the diameter was maximal, could be found at some focal level within the thickness of the section (10). The sections used for morphometric measurements were cut with the microtome set for 2 μm section thickness. However, the actual average thickness, T, was measured by the fold method of Small (27), and found to be 1.76 μm ± 0.03 SEM. The height, h, of the smallest recognizable cap section was arbitrarily estimated to be one-third of the section thickness, that is, ~10% of the nuclear diameter, although it might have varied somewhat with staining intensity. The calculation of numerical density by the Floderus equation is only accurate in the strictest sense if the nuclei are spherical. Even though the nuclei observed by light microscopy in the present study were commonly somewhat ellipsoidal in shape, their axial ratio was not observed to reach levels that would produce serious error (15; also see Fig. 3 of reference 5).

The volume of an average Leydig cell was derived by dividing the volume density by the numerical density.

ELECTRON MICROSCOPY: Leydig cells occur in scattered groups and occupy only a small percentage (2.7%) of the section area, making it impractical to sample random electron microscope sections systematically. The few Leydig cells were often behind grid bars in a particular section. Consequently, micrographs were taken wherever Leydig cells could be found in several consecutive sections mounted on the same grid, but care was taken to insure that no particular cell was represented in more than one micrograph taken from these serial sections at a given magnification. Micrographs were taken at both × 3,600 and 24,000, and the higher power micrographs always came from the central part of an area that had been taken at lower power (which could possibly have biased the sampling somewhat).

Morphometric analysis was carried out on 50 electron micrographs of Leydig cells at × 10,800 (from × 3,600 negatives), and 50 more at × 72,000 (from × 24,000 negatives) for each of three animals, and for the other animal (No. 3 in Table I), 100 micrographs were analyzed at each magnification. These micrographs came from 10 different tissue blocks for each of the three animals, and 20 tissue blocks for the remaining animal (five micrographs per tissue block).

The lower magnification views were used to estimate volume density of organelles, and also the surface density of Leydig cell plasma membranes. The higher magnification views provided surface density measurements of organelles, and also an additional estimation of organelles volume density, for comparison with the values obtained at lower magnification. The technique was similar at both magnifications. For volume density, a transparent overlay bearing a double-lattice grid (Fig. 5) was placed on the electron micrograph, and the fraction of points occurring within the structure of interest was determined and then compared to the total number of points lying within Leydig cells. The grid contained 432 total test points, of which 108 coarse points were defined by intersecting dark lines, making a ratio of 4:1. The coarse points were used on lower power micrographs to measure the volume density of mitochondria, while the full number of points were employed for other volume determinations. The results of the above counts, giving organelle volume per Leydig cell volume, were then multiplied by the volume density of Leydig cells to yield the final volume density of the organelle (which is the organelle volume per unit volume of testis tissue).

For the measurement of surface density (S_V), a multipurpose grid (illustrated in Fig. 6) was used, containing 45 lines of known length, whose intersections with pertinent surface contours on the micrograph were recorded. For convenience, the equation used to calculate the surface density (28) was:

$$S_V = 4I/P_T \cdot z,$$

where I is the number of intersections, P_T is the number of line end points that occur over Leydig cells, and z is the average

TABLE I

Basic Data on Rats Studied

Animal No.	Body Weight	Testis weight after perfusion	Estimated in fresh testis*	
			Weight	Volume
	g	*g*	*g*	cm^3
1	365	2.024	1.69	1.63
2	255	1.652	1.38	1.32
3	265	1.836	1.53	1.47
4	345	2.167	1.81	1.74
Mean	307.5	1.920	1.60	1.54
±SE	±27.8	±0.112	±0.094	±0.092

* Based on data presented in Materials and Methods, showing that the rat testis increases in volume on the average by a factor of 1.201 ± 0.023 (SE) (n = 5), after perfusion-fixation; the specific gravity of fresh and fixed testes is essentially the same (1.040 ± 0.004 and 1.039 ± 0.007, respectively, n = 5).

length of individual lines in terms of the scale of the micrograph. Here again, the counts yield organelle surface area per Leydig volume, and it was necessary to multiply these results by the volume density of Leydig cells to obtain the surface density of the organelle (which is the organelle surface area per unit volume of testis tissue).

The estimation of numerical density for approximately spherical organelles such as lysosomes, peroxisomes, multivesicular bodies, and lipid droplets was carried out, using the equation:

$$N_V = (K/\beta)\cdot(N_A^{3/2}/V_V^{1/2}),$$

as described by Weibel and Bolender (28). The shape factor β was determined from the average axial ratio, using the graph on page 250 of that reference. The coefficient K for relative size distribution was calculated from Eq. 11, page 642, of reference 1.

Electron micrographs of 0.5-μm thick sections indicated that mitochondria of rat Leydig cells were generally cylindrical in shape, with two hemispherical ends. It was not possible to find the mean length of the mitochondria by measurements on these sections, nor on conventional thin sections, because one sees mostly profiles in various planes of section. Assuming that most mitochondria are cylinders with hemispherical ends, then the mean volume $V = \pi D^2(3L + 2D)/12$, while the average surface area would be $S = \pi D(L + D)$, where L is the average length of the mitochondrion (not counting the hemispherical ends), and D is the average diameter. The diameter D can be obtained directly by measuring the minor axis of profiles on conventional electron micrographs, as the shorter axis is the correct diameter regardless of the plane of section (the only exception would be dumbbell-shaped profiles, arising from a section through a bend in the mitochondrion). The ratio of V to S is the same as the ratio of volume density V_V to surface density S_V for Leydig mitochondria in the tissue as a whole, both of which are known. Since V/S is therefore known and D can be measured, then $V/S = D(3L + 2D)/12(L + D)$ can be solved for L, and $L + D$ equals the total length of an average mitochondrion. The values for V and S can then be found from the original equations given above. To find the average number of mitochondria per cell, the total mitochondrial volume per cell is then divided by V, the volume of the average mitochondrion. An approximate numerical density of Leydig mitochondria is then determined by multiplying the average number of mitochondria per cell by the numerical density of Leydig cells.

Systematic Errors of Measurement

Several factors which might affect the primary data were taken into account. Estimated correction factors are shown in Tables II and III. Data throughout the paper have been corrected by these factors.

FIXATION: The effect of fixation on the testis was assessed (Table I). Specific gravity of both fresh and fixed testes was determined by flotation of the testes in several sucrose solutions of known concentration. The volume was then obtained by dividing weight by specific gravity. Testes fixed by perfusion with 0.1 M collidine-buffered 3% glutaraldehyde increased in volume by 20.1%, compared to fresh tissue. This was shown by comparing the volume of the fixed left testis with that of the unfixed right testis, taking into account that the normal left testis is smaller than the right by a factor of 0.984 in adult animals (average of weight measurements on four animals). The volume increase seen during fixation resulted from perfusion pressure, and presumably involved the expansion of blood vessels and lymphatic spaces, which are the most likely expandable compartments.

DEHYDRATION AND EMBEDDING: Tissue volume was estimated by measuring the dimensions before dehydration and after polymerization of the embedding medium on both light and electron microscope blocks (Table II). Tissue in blocks for light microscopy had their volume reduced by 22.2%, while those for electron microscopy were reduced by 11.4%. Osmication seemed to lessen the degree of shrinkage caused by dehydration. Because both light and electron micrographs showed no appreciable widening of the interstitial space, some of the fluid which had filled the dilated lumens of blood vessels and expanded interstitial

TABLE II

Correction Factors for Sample Preparation

	Volume	Area	Length
Glycol methacrylate			
Fixation	×1.201*		
Dehydration	×0.778		
Sectioning	×1.000	×1.147‡	×1.071
Correction factor			0.955§
Epon			
Fixation	×1.201		
Dehydration	×0.886		
Sectioning	×1.000	×0.893	×0.945
Printing	—	×1.022	×1.011
Correction factor			1.026§

* Volume increased by 20.1% after perfusion-fixation.

‡ Area increased by 9.6% after sectioning ($1.201^{2/3} \times 0.778^{2/3} \times 1.147 = 1.096$).

§ Correction factors were applied only to surface density and diameter of structures.

TABLE III

Correction Factors for Section Thickness

Organelles	Model, shape factors	Correction factors (K_v) for volume density	Correction factors (K_s) for surface density
Nucleus	Sphere, volume density: $g = 0.28$, $\rho = 0.3$, surface density: $g = 0.008$, $\rho = 0.15$	0.709	0.991
Mitochondria	Tubule, $d = 350$ nm, $L = 2400$ nm, $\lambda = 6.9$, $g = 0.15$	0.957	
Outer and inner membranes (excluding cristae)	(same as above)		0.972
Cristae	Tubule, $d = 28$ nm, $L = 290$ nm, $\lambda = 10.4$, $g = 1.82$		0.479
Peroxisomes	Sphere, $g = 0.115$, $\rho = 0.3$	0.855	0.881
Lysosomes	Sphere, $g = 0.098$, $\rho = 0.3$	0.874	0.898
Multivesicular bodies	Sphere, $g = 0.103$, $\rho = 0.3$	0.869	0.893
Lipid droplets	Sphere, $g = 0.053$, $\rho = 0.3$	0.929	0.947
SER	Tubule, $d = 64$ nm, $L = 346$ nm, $\lambda = 5.4$, $g = 0.80$	0.549	0.628
RER	Disk, $d = 46$ nm, $D = 580$ nm, $\delta = 12.6$, $g = 1.11$	0.636	0.923

Correction factors for section thickness, calculated according to Weibel and Paumgartner (29) from the shape factors indicated (those available in the text are not listed). The section thickness T was 51 nm for all calculations except nuclear volume, where $T = 1.76$ μm.

lymph spaces after perfusion fixation must have left the tissue in the process of dicing and dehydration.

SECTIONING: When sections from blocks embedded in GMA were floated on water, their width increased by 9.4%, while their length increased by 4.8% (the latter figure being smaller because of compression). Then, the area of section increased by 14.7%. On the other hand, sections from Epon-embedded blocks showed a reduction of length by 10.7%, while the width remained unchanged, the section area being reduced by 10.7%.

Because the spreading or compression of section in sectioning should cause a decrease or an increase of section thickness, the exact thickness of both light and electron microscope sections was determined by the fold method of Small (27). The average thickness of light and electron microscope sections was found to be 1.76 μm ± 0.03 SEM and 51 nm ± 0.52 SEM, respectively.

OPTICAL DISTORTION: The light microscope, electron microscope, and enlarger for printing presumably have some optical distortion, as suggested for the electron microscope by Loud et al. (21). However, such distortions on the equipment used in the present study (Zeiss light microscope, JEOL 100B EM, and Durst S-45EM enlarger) are probably of small enough magnitude to be negligible.

PRINTING PAPER: Printing papers increased in area by 2.2% after drying.

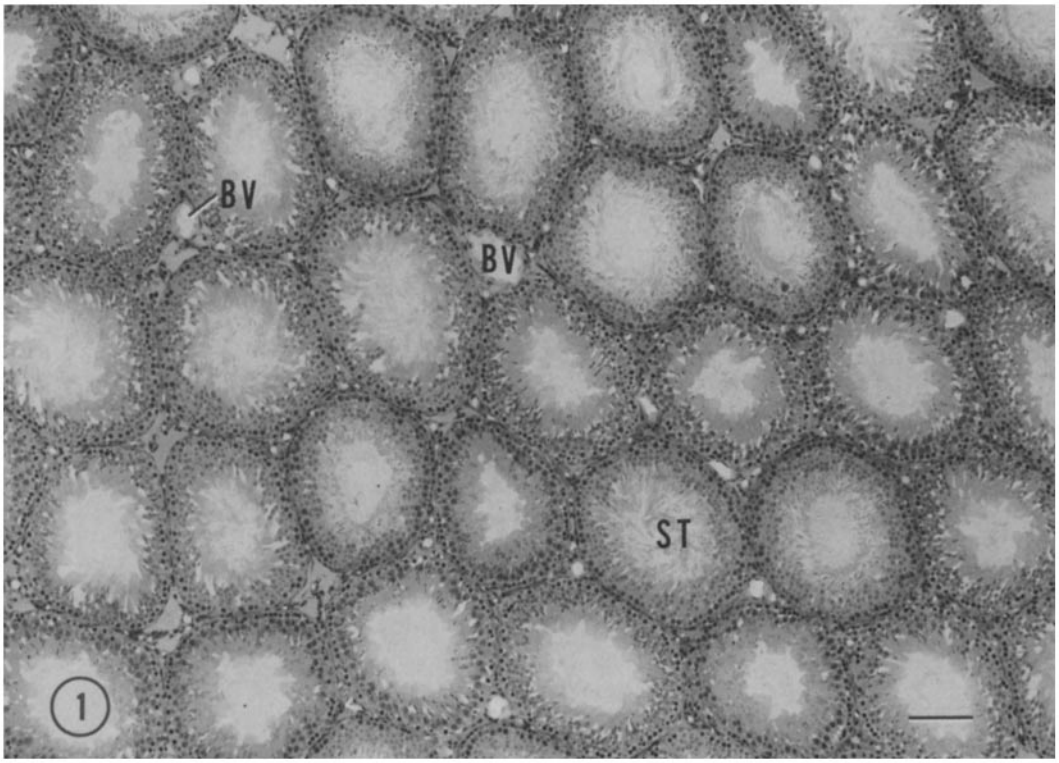
EFFECT OF SECTION THICKNESS: The basic equations of stereology utilized in this study are strictly accurate only for sections that are infinitely thin. Because our electron microscope sections have finite thickness, the volumes and surface areas measured on them tend to be overestimated. In a recent paper, Weibel and Paumgartner (29) discuss this problem and provide methods for deriving appropriate correction factors, depending on the size and shape of the structures being measured and the thickness of the sections. We have utilized their approach to develop correction factors (Table III) for both volume and surface densities of organelles in our electron microscope preparations, and for volume density of nuclei in our light microscope preparations. The data in Table IV and elsewhere in the text have undergone these corrections.

SIZE AND LOCATION OF SAMPLE: The same counts were often made on organelles in both low- and high-magnification electron micrographs. The final data for volume densities generally were taken from counts at lower magnification, because the sampling problems increase considerably at higher magnification for organelles that are not especially numerous, such as lysosomes, peroxisomes, multivesicular bodies and lipid droplets. For these structures, the surface density measurements (which were done only at higher magnification) were assumed to be affected by the same sampling problems. These surface counts were corrected by a factor equal to the ratio of the volume density of the structure counted at lower magnification to the volume density at higher magnification.

RESULTS

The quality of preservation obtained in this perfusion-fixed material makes it favorable for morphometric study. At the light microscope level (Figs. 1 and 2), tissue relationships are well maintained, and there is little artifactual expansion of the interstitial tissue. In electron micrographs (Figs. 3 and 4) the structure and relationships of organelles are well preserved and the membranes stand out clearly, facilitating morphometric analysis. Figs. 5 and 6 illustrate the two types of overlays used for the EM counts.

A detailed presentation of the morphometric



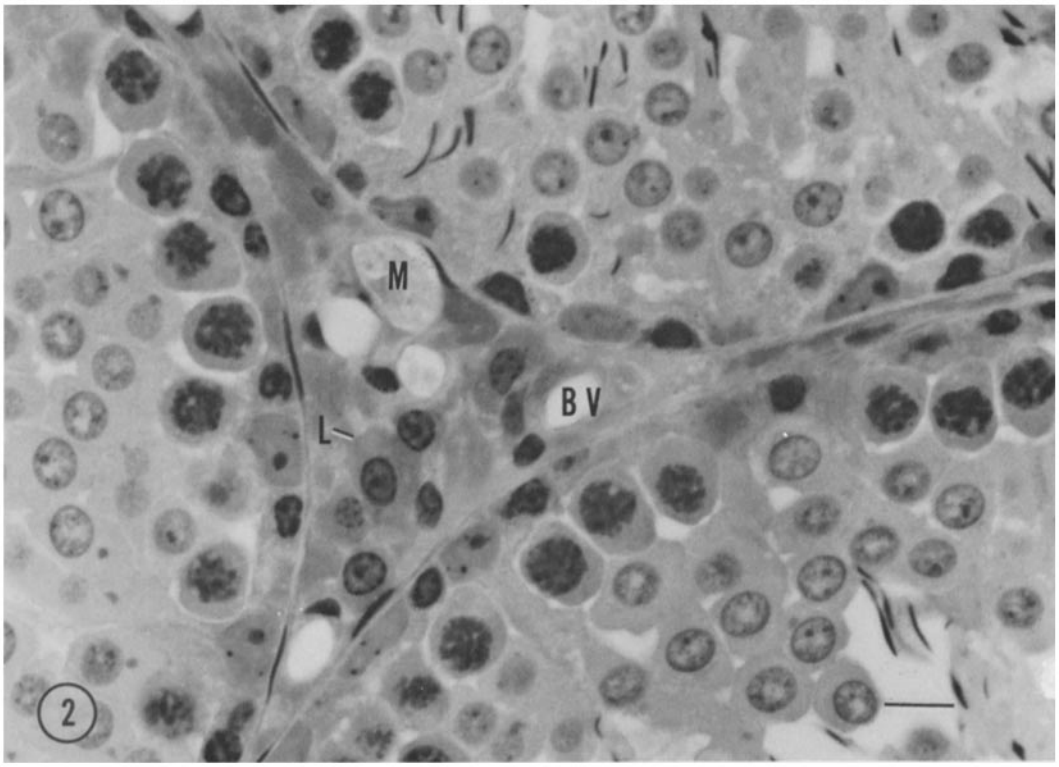


FIGURE 1 Light micrograph of rat testis fixed by perfusion and embedded in glycol methacrylate. The interstitial space, lying between seminiferous tubules (*ST*), is well maintained, without obvious artifactual expansion. Blood vessels (*BV*) are empty and somewhat swollen, as a result of the perfusion. Bar, 100 μm. × 84.

FIGURE 2 Interstitial tissue from the same section as Fig. 1. Leydig cells (*L*) are easily distinguished by their characteristic nucleus and dark red cytoplasm. The cytoplasm of macrophages (*M*) appears pale, because the trypan blue they had contained was extracted during tissue preparation for microscopy. Capillaries (*BV*) are also apparent. Bar, 10 μm. × 920.

results is given in Table IV, and a graphic summary of volume percentages is provided in Fig. 7. The following description will cite only the more important of these results.

In the fresh state, the testes used in this study weighed an average of 1.60 g and had an average volume of 1.54 cm^3 (Table I). As the mean specific gravity for normal fresh testis is 1.040, 1 cm^3 of normal fresh testis weighs 1.040 g, and 1 g is equivalent to 0.962 cm^3. These conversions are essentially the same in fixed tissue, where the specific gravity is 1.039.

In a whole testis, including capsule (tunica albuginea), 82.43% of the volume is occupied by seminiferous tubules, 15.64% by the interstitial tissue, and 1.93% by the capsule.When decapsulated, the testis tissue consists of 84.0% seminiferous tubules and 16.0% interstitial tissue, while the Leydig cells make up 2.8% of the total volume. This latter value may be somewhat low, as glutaraldehyde fixation causes a slight shrinkage of the cells.

There are 22.04 million Leydig cells/cm^3 of testis tissue, which means that a 1.60-g testis (average in the present study) contains 34 million, and an average 300-g rat would have ~70 million Leydig cells in both testes. An average Leydig cell has a volume of 1,209 μm^3 and a surface area of 1,517 μm^2. Again, the volume may be low because of slight glutaraldehyde-induced cell shrinkage. If a Leydig cell of that volume were spherical, it would be 13.2 μm in diameter and would have a surface area of 549 μm^2. However, the actual surface area is 2.8 times that amount, which reflects the irregular shape and extensive surface processes of these cells.

Nuclei of Leydig cells occupy 0.33% of testicular volume. In an average Leydig cell, the nucleus has a volume of 150 μm^3, which is 12.4% of cellular volume. The nucleus is approximately ellipsoidal, with an average axial ratio of 1.37 and a mean diameter of 6.34 μm. The surface area of an average nucleus is 149 μm^2.

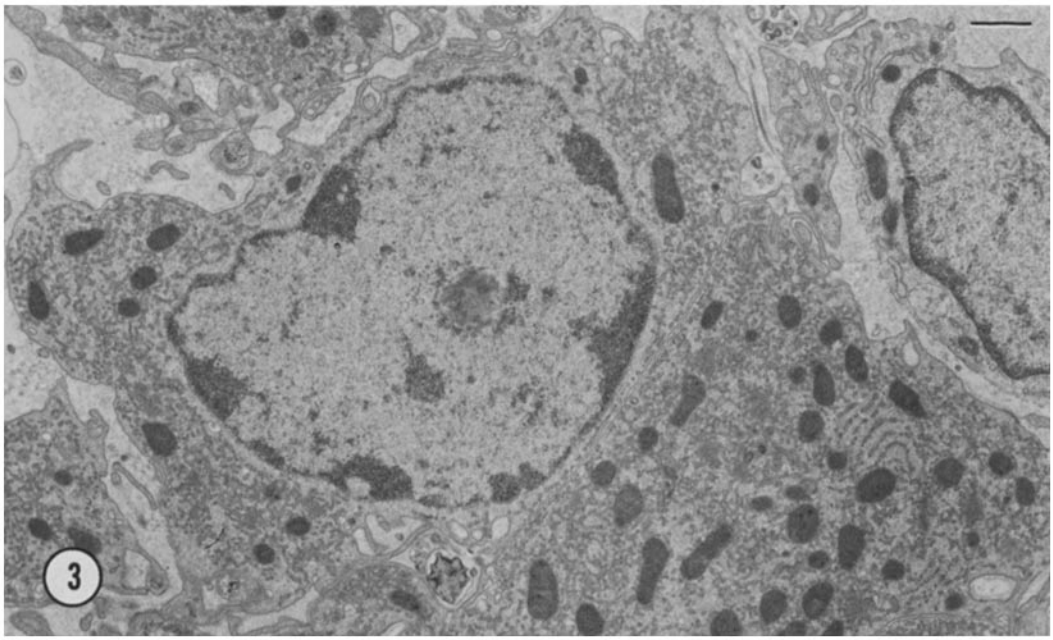
The SER is the most abundant organelle, occupying a volume of 138 μm^3 in the cytoplasm of an average cell, and thus comprising 13.0% of cytoplasmic volume or 11.4% of cell volume. These volumes were measurements of the organelle itself (membrane plus contents), and do not refer merely to regions of the cell in which SER predominates. The SER of Leydig cells constitutes ~0.3% of testicular volume. The membrane surface area of the SER is vast. There are 2,305 cm^2 or 0.23 m^2 of SER membranes in each cm^3 (very nearly 1 g)[1] of testis tissue. The individual Leydig cell contains an average of 10,458 μm^2, equivalent to a square that is 0.10 mm on a side. The SER of a Leydig cell therefore has about 6.9 times as much membrane surface area as is present in the plasma membrane, and constitutes ~60% of the membrane surface area found throughout the whole cell.

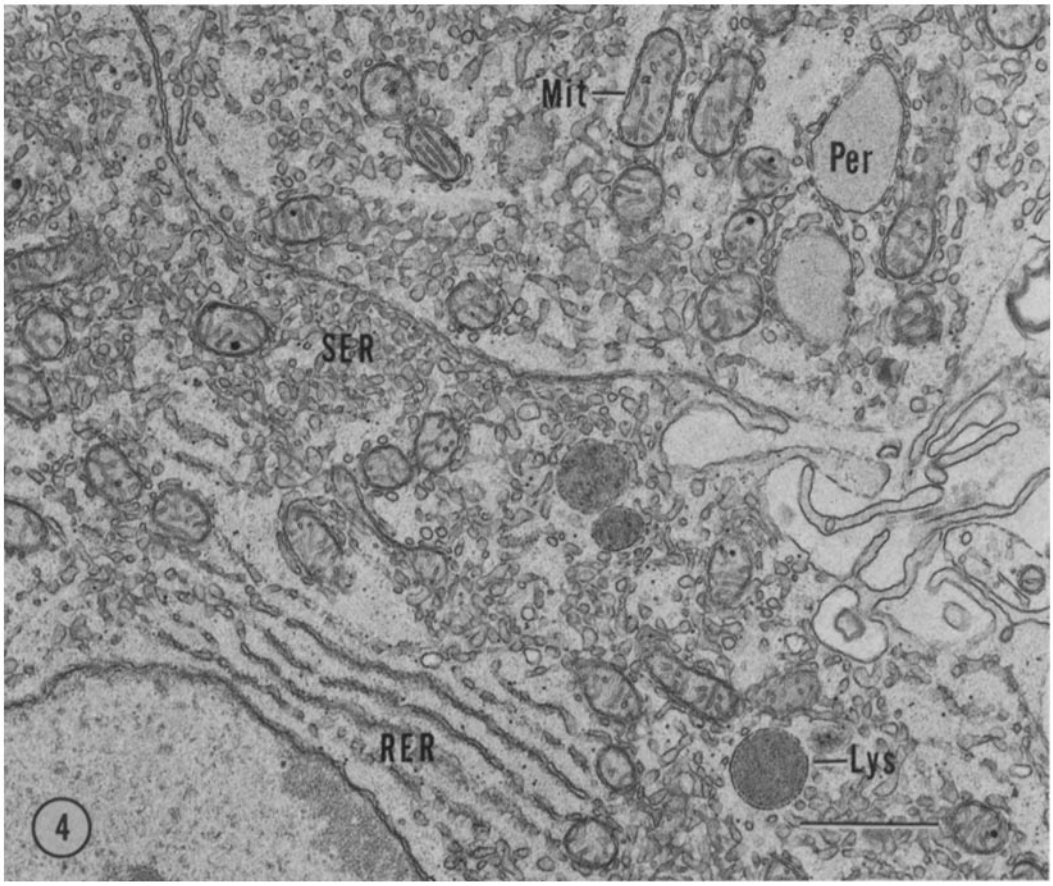
In this study, the Golgi complex is included

[1] It is customary in morphometry to express volumes in terms of "cm^3." However, for biochemical applications of morphometric data it is generally more useful to have volume information in terms of "grams." The two values "cm^3" and "gram" are approximately interchangeable, because the specific gravity of fresh testis tissue from rats is near unity (1.040 ± 0.004 SEM). When one is dealing with volume densities (volume of a structure per unit volume of tissue), the values for "cm^3/cm^3" and "g/g" are identical, as the specific gravity factors cancel out. Wherever desirable, therefore, it is possible to express the volume densities directly in terms of grams. As an example, from Table IV there would be ~3.3 mg of Leydig cell nuclei in 1 g of rat testis, on the average. Surface and numerical densities can be converted to a per gram basis by dividing the cm^3 value by 1.040. For most practical purposes these figures are close enough so conversion is unnecessary.

FIGURE 3 Low power electron micrograph of Leydig cells from a perfusion-fixed testis. Structural features are well maintained. The small cell at upper right is probably a fibroblast. The tissue was prepared as described in Materials and Methods, except that it was postfixed with phosphate-buffered OsO_4, and the material was block-stained with aqueous uranyl acetate. Bar, 1 μm. × 8,200.

FIGURE 4 Cytoplasm of two Leydig cells, showing smooth endoplasmic reticulum (*SER*), rough endoplasmic reticulum (*RER*), mitochondria (*Mit*), peroxisomes (*Per*), and lysosomes (*Lys*). A nucleus is at lower left. This material was postfixed in OsO_4-ferrocyanide, as described in Materials and Methods, and is typical of micrographs used for counting. This postfixation is advantageous for morphometry, as it causes membranes to stand out clearly, decreases the density of the mitochondrial matrix, and permits easy differentiation between the pale-staining peroxisomes and densely staining lysosomes. However, ribosomes stain poorly, making it more difficult to distinguish the sparse RER of these cells. Bar, 1 μm. × 19,400.





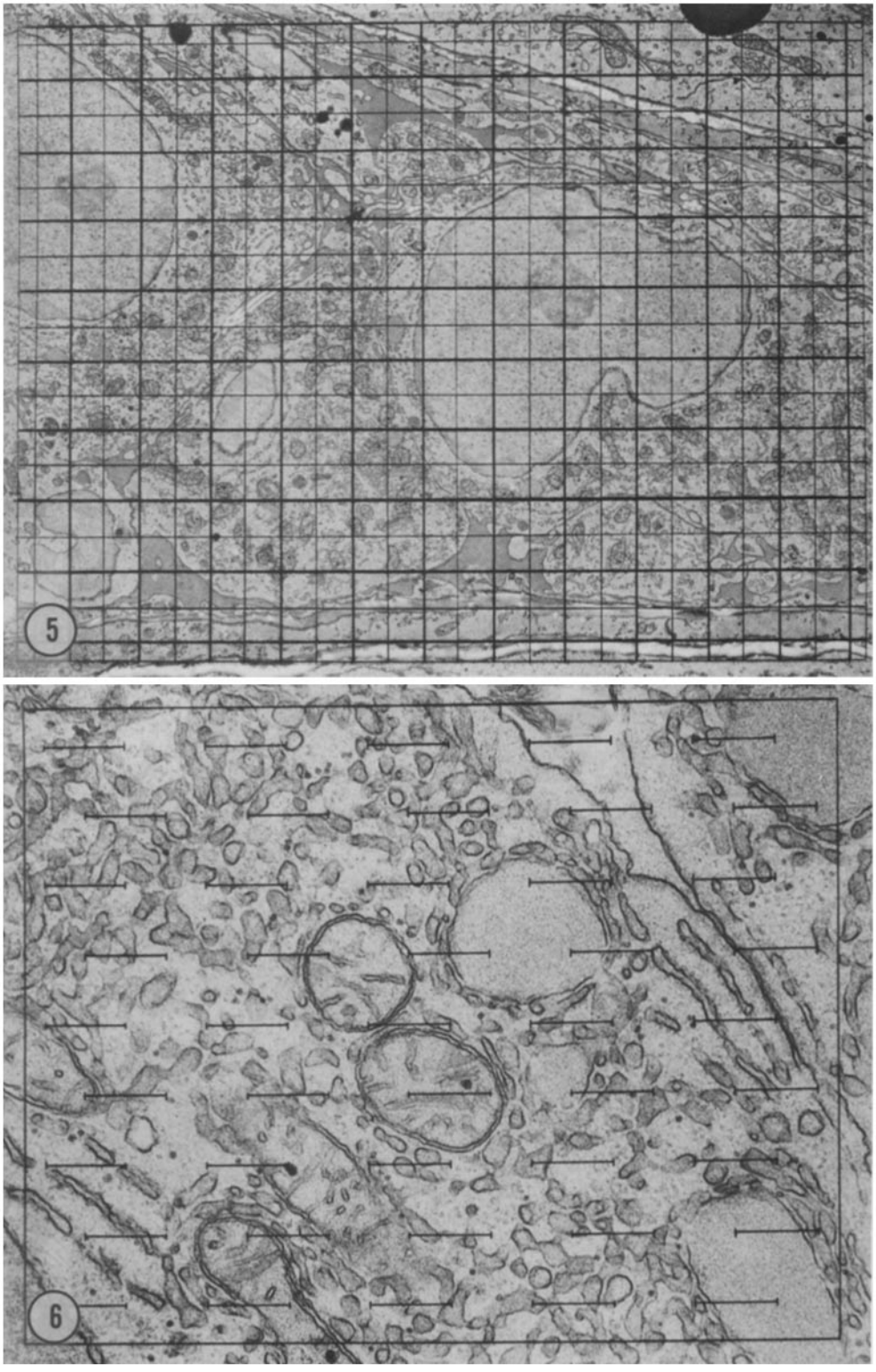


FIGURE 5 Double-lattice grid placed over micrograph of Leydig cells. This overlay was used for volume density measurements. The intersections of lines constitute counting points, of which there are a total of 432. Included in this number are 108 "coarse" points, defined by the intersections of dense lines. × 5,900.

FIGURE 6 Multipurpose grid superimposed on a region of Leydig cell cytoplasm. The grid contains 45 test lines and 90 end points. This overlay was used for obtaining surface densities as well as volume densities of organelles. × 38,500.

TABLE IV

*Normal Values of Morphometric Parameters for Rat Testis and Leydig Cells**

Component	Parameter	Mean value/cm³ tissue	SEM	SE as % of mean	Per average testis‡	Per average Leydig cell§
Capsule	V	0.0193	0.0028	14	0.0297	
Seminiferous tubules	V	0.8243	0.0009	0.1	1.2694	
Interstitial tissue	V	0.1564	0.0030	2	0.2409	
Leydig cells	N	22.04×10^6	1.03×10^6	4.7	33.94×10^6	1
	V	0.02665	0.00126	5	0.04104	1,209
	S	334.4	12.8	4	515.0	1,517
Nuclei	V	0.00330	0.00018	5	0.00508	150
	S‖	32.75	1.06	3	50.44	149
Cytoplasm	V	0.02335	0.00119	5	0.03596	1,059
Endoplasmic reticulum	V	0.00324	0.00025	8	0.00499	147
	S	2,424	178	7	3,733	10,998
Smooth (includes Golgi)	V	0.00305	0.00025	8	0.00470	138
	S	2305	177	8	3550	10,458
Rough	V	0.00019	0.000007	4	0.00029	9
	S	118.8	6.66	6	183.0	539
Mitochondria	N	13.71×10^9	0.89×10^9	6	21.11×10^9	622
	V	0.00301	0.00010	3	0.00464	137
Outer membrane	S	361.6	28.1	8	556.9	1,641
Inner membrane (including cristae)	S	643.6	25.1	4	991.1	2,920
Peroxisomes	N	6.87×10^9	0.55×10^9	8	10.58×10^9	312
	V	0.00033	0.00005	15	0.00051	15
	S	43.09	5.78	13	66.36	196
Lysosomes	N	2.86×10^9	0.36×10^9	13	4.40×10^9	130
	V	0.00015	0.000003	2	0.00023	7
	S	19.31	0.73	4	29.74	88
Multivesicular bodies	N	1.45×10^9	0.18×10^9	12	2.23×10^9	66
	V	0.00007	0.000004	6	0.00011	3
	S	9.45	0.79	8	14.55	43
Lipid droplets	N	8.3×10^7	2.2×10^7	26	12.8×10^7	4
	V	0.00006	0.00002	33	0.00009	3
	S	3.58	0.96	27	5.51	16
Cytoplasmic ground substance	V	0.01649	0.00086	5	0.02539	748
Golgi area¶	V	0.00038	0.00003	9	0.000585	17

Dimensions of the parameters: (Note: It is possible to substitute "gram" approximately for "cm^3" throughout these parameters, as the specific gravity of testis tissue [=1.040] is near unity.)

Number (N): No./cm^3, No./testis, and No./cell, respectively.

Volume (V): cm^3/cm^3, cm^3/testis, and μm^3/cell, respectively.

Surface area (S): cm^2/cm^3, cm^2/testis, and μm^2/cell, respectively.

* Average values from four animals, corrected as described in the text.

‡ Obtained by multiplying the value per cm^3 tissue by 1.54, the mean volume of four testes from the animals studied.

§ Obtained by dividing the value per cm^3 tissue by the Leydig cell number per cm^3 tissue.

‖ Also included in the surface density of the RER.

¶ Regions of cytoplasm containing Golgi stacks.

with the SER for counting purposes, as Golgi membranes are not abundant and are often difficult to distinguish from the SER in oblique sections. Also, the identity of membranes located at the periphery of Golgi stacks was not always certain. However, a separate count shows that areas of the cytoplasm in which Golgi stacks occur constitute ~1.6% of the cytoplasmic volume.

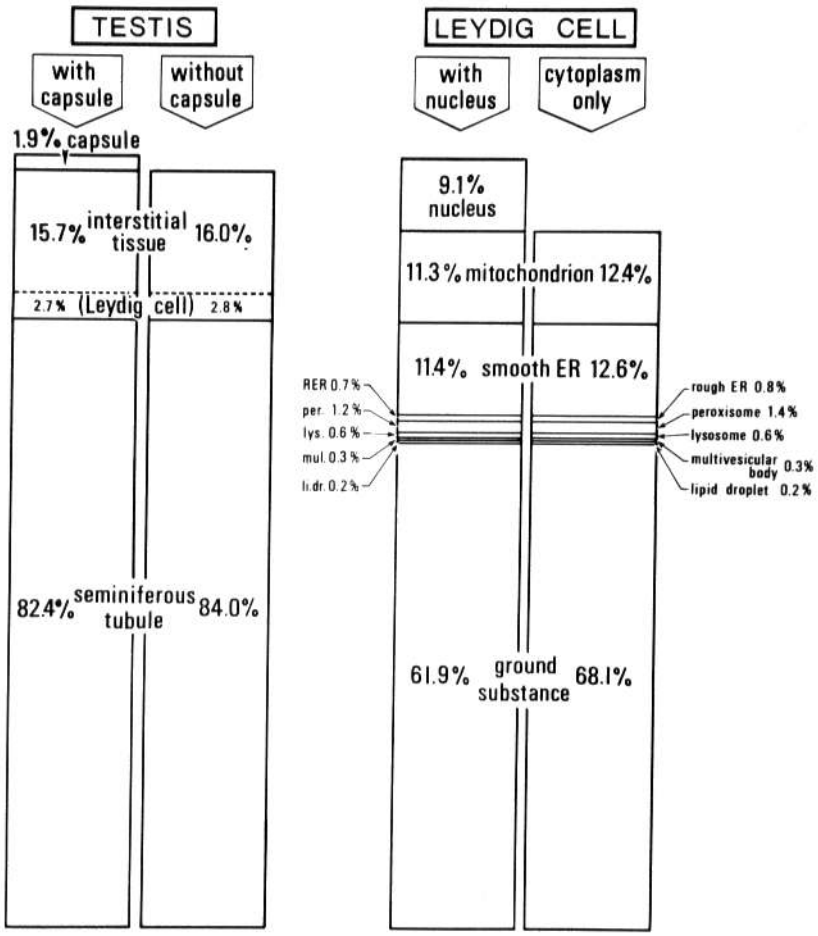


FIGURE 7 Volumetric composition of testis tissue and of Leydig cells.

The RER is distinguished primarily by the characteristic arrangement of its membranes in flattened cisternae that are more widely separated from one another than those of Golgi stacks. As the tissue in our preparation has been postfixed with OsO_4-ferrocyanide, the ribosomes are virtually unstained, and therefore cannot be used as a criterion in identifying the RER. As a result, some RER may have passed undetected, and this organelle is thus probably underestimated in the present study. At best, it is poorly developed in steroid-secreting cells. In Table IV, the volume density of the perinuclear cisterna has been included with that of the RER, and the surface density of the external, ribosome-studded membrane of the nucleus has been counted as part of the RER. The volume occupied by RER (membrane plus lumen) in a Leydig cell is 8 μm^3, which is 0.8% of cytoplasmic volume. The surface area of RER membranes is 119 cm^2/cm^3 of tissue, and the surface ratio of SER to RER is thus about 19.

The mitochondria in rat Leydig cells are not so large or so complex in internal structure as those seen in Leydig cells of some other species, or as those in some other steroid-secreting tissues (see references 8 and 9 for reviews). In rat Leydig cells, the average mitochondrion was 0.35 μm in diameter and 2.4 μm long, the ratio of length to diameter thus being 6.9. The average Leydig cell contained ~622 mitochondria, occupying a volume of 137 μm^3, which is 12.9% of the cytoplasm or 11.3% of cell volume. The mitochondria of Leydig cells made up 0.3% of testis volume, which means that 1 g of testis would contain ~3.0 mg of Leydig mitochondria. The surface area of the outer mitochondrial membranes is 362 cm^2/cm^3 tissue, while that of the inner membrane (including the cristae) is 644 cm^2/cm^3 tissue. Thus, the inner

membrane, site of certain important enzymes of steroid biosynthesis (see Discussion), has a surface area 1.8 times greater than that of the outer membrane of the mitochondrion, but its surface area is less than that of the SER (which contains the majority of steroidogenic enzymes) by a factor of 3.6. In an average Leydig cell there are 1,640 μm^2 of membrane surface area in the outer mitochondrial membranes and 2,920 μm^2 in the inner mitochondrial membranes.

The peroxisomes, or microbodies, found in steroid-secreting cells are usually small, and are therefore called "microperoxisomes" (23). However, in rat Leydig cells they are unusually large, with a mean diameter of 0.46 μm. They are somewhat irregular in shape, commonly exhibiting an oval profile in sections. They have been identified cytochemically by Reddy and Svodboda (24). In the present material (Fig. 4), postfixation with OsO_4-ferrocyanide stained lysosomes rather densely, whereas peroxisomes were only slightly stained, making it comparatively easy to distinguish these two organelles from one another. There are ~312 peroxisomes in an average Leydig cell, occupying 1.2% of the cell volume. Each cubic centimeter of testis tissue contained about 6.9 billion Leydig peroxisomes, occupying 0.03% of tissue volume.

Lysosomes are usually spherical in shape, with an average diameter of 0.48 μm, and typically stain densely with the OsO_4-ferrocyanide poststaining used in this study. Primary lysosomes, with homogeneous matrix, are most common, but residual bodies and occasional autophagic vacuoles are also seen. Lysosomes constitute only 0.6% of Leydig cell volume.

Multivesicular bodies average 0.43 μm in diameter and comprise 0.3% of cell volume. These organelles are recognized by their content of small vesicles, although in multivesicular bodies occurring near the Golgi complex the vesicles may be sparse and may not be included in the plane of section. The limiting membrane of this organelle is noticeably thicker than that of nearby SER, and some segments of the membrane may be "bristle-coated" (26).

In adult laboratory rats, the Leydig cells characteristically contain very few lipid droplets, averaging only about four per cell, occupying a mere 0.2% of cell volume; the lipid droplets have a mean diameter of 1.06 μm. This is in contrast to the condition in fetal and prepubertal rats, where the Leydig cells contain abundant large lipid droplets (9).

The cytoplasmic ground substance, or background cytoplasm lying between major organelles, constitutes 61.9% of cellular volume, or 70.6% of cytoplasmic volume. It contains free ribosomes and polysomes, microfilaments, microtubules, and other small cytoplasmic components, as well as soluble materials constituting the "cytosol" of fractional centrifugation.

DISCUSSION

This study provides morphometric data on rat Leydig cells that can be correlated with available biochemical information, thus contributing to a quantitative cell biology for these cells.

Several recent articles have appeared on Leydig cell morphometry, utilizing a variety of stereological approaches. Kerr et al. (18) compared the volume of Leydig cells in the testes of normal and cryptorchid rats, and found a distinct increase in the volume of the cells and their organelles in the cryptorchid. To obtain cell volume, they made planimetric measurements of cell areas in sections, calculated an average cell diameter by extrapolating the area to a circle, and then used the diameter to calculate cell volume, assuming that the cells were approximately spherical in shape. Organelle volume was calculated from the percentage of cytoplasmic volume occupied by the organelle, determined by point counting on electron micrographs. The method could be effective for showing relative changes in cell and organelle volumes, assuming that cell shape does not change. The absolute values for the volumes differed considerably from those obtained in the present study. Bergh and Damber (3) and Bergh and Helander (4) studied the Leydig cells in rats rendered unilaterally cryptorchid at birth and found that by the time the animals reached maturity, the Leydig cells were smaller in the abdominal testis than in the control. The total number of Leydig cells was about the same in both testes, but since the seminiferous tubules were greatly reduced in the cryptorchid testis, the Leydig cells there occupied a considerably higher proportion of testicular volume (i.e, their volume density was greater). These authors used point counting for volume densities and derived a total Leydig cell mass per testis by multiplying Leydig volume density times testicular weight. The average volume of an individual Leydig cell was estimated as a ratio between Leydig

volume density and a numerical density of Leydig nuclei derived from nuclear counts. The total number of Leydig cells per testis was then calculated by dividing the total Leydig cell mass by the mean Leydig cell volume. The numerical density of Leydig cells for normal testes given in their results was about half the figure obtained in the present study, and the volume density of Leydig cells was also somewhat lower than that given here. Kaler and Neaves (16) found that human Leydig cells become less numerous in older individuals. These authors determined Leydig volume density by point counting on Bouin-fixed and methacrylate-embedded tissue, and multiplied this value by testicular weight to obtain total Leydig volume per testis. They calculated the volume of an average Leydig cell from measured cell diameters, assuming the cells to be spherical. The total Leydig cell number per testis was then derived by dividing the total Leydig volume per testis by the volume of an individual Leydig cell. The average numerical density of Leydig cells in a 20-yr-old man, calculated from their data, would be about 16 million/g of testis, which is not far from the figure described for rats in the present study. Kothari et al. (19) have measured total Leydig cell volume per testis for several common animals. References to earlier literature can be found in the above papers and in reference 10.

In another paper from this laboratory, Christensen and Peacock (10) have studied the change in Leydig cell numbers under chronic treatment with excess hCG in adult rats. That morphometric study was carried out at the light microscope level on tissue fixed with Bouin's fixative, embedded in paraffin, and section at ~7 μm thickness. The stereological procedures were similar to those used in the present paper. The results showed that the number of cells increased by a factor of 3 over 5 wk of treatment. In spite of the considerable technical differences in specimen preparation between that study and the present one, the number of Leydig cells per cubic centimeter of testis in normal animals was similar, being 18.6 million compared to 22.0 million in the present study. The present figure is probably more accurate, since the histological preparations used in the other study produced some artifactual expansion of the interstitial space, which could explain the lower numerical density. Christensen and Peacock (10) discussed the difficulties involved in obtaining accurate numerical densities, and this topic will not be repeated here.

Free and Tillson (14) reported the in vivo secretion rate of testosterone in conscious and in halothane-anesthetized rats as 4.2 ± 0.6 and 6.7 ± 1.2 ng/g testis per min, respectively. The authors considered the value obtained under anesthesia to be more characteristic of the unstressed animal. We will utilize the secretion rate from their study, even though they used larger rats (473 g average), which may secrete testosterone at a somewhat lower rate than the younger rats used in our study (307 g average); see their paper (14) for a discussion of this point. Dividing the secretion rate from their study by the number of Leydig cells per gram of testis, derived from our counts, it can be shown that an average individual Leydig cell secretes ~0.44 pg of testosterone/d, or ~10,600 molecules of testosterone/s. The corresponding values published by Christensen and Peacock (10), based on the number of Leydig cells per cubic centimeter found in their study, were 0.5 pg of testosterone/d or 12,500 molecules/s.

It is possible to extend these correlations with secretion rate to the subcellular level. A majority of the steroidogenic enzymes in Leydig cells are bound to membranes of the SER, although some are located on the inner membrane and cristae of mitochondria (see reference 8 for review). To summarize, acetyl-CoA, arising within mitochondria from fatty acids or glucose, enters the cytoplasm and is converted by several soluble enzymes and one SER-bound enzyme to farnesyl pyrophosphate. Subsequent steps to cholesterol are catalyzed by enzymes situated on membranes of the SER. To begin its conversion to testosterone, cholesterol first enters a mitochondrion, where its side chain is cleaved by enzymes on the inner membrane and cristae. The remaining steps to testosterone are catalyzed by SER-bound enzymes. The SER and inner mitochondrial membranes thus are of fundamental importance as enzyme sites for steroid biosynthesis.

Because the SER and inner membranes of mitochondria play a central role in steroidogenesis, it is not surprising that they are abundant in Leydig cells, as shown in the morphometric results of the present study. Membranes of the SER in a Leydig cell present a large surface area for enzymes, being 6.9 times as extensive as plasma membranes. The inner membranes of mitochondria (including cristae) within a Leydig cell have a surface area 1.9 times that of the plasma membrane. Using the secretion rate data described above, it can be calculated that each square cen-

timeter of SER produces ~4.2 ng of testosterone/d or 101 million molecules/s. The corresponding values for each square centimeter of inner mitochondrial membrane are 15 ng/d or 362 million molecules/s. In other words, it takes 1.0 μm^2 of SER and 0.3 μm^2 of mitochondrial inner membranes on the average to produce testosterone at a rate of one molecule/s.

The findings of this study may also be used to estimate the rate of cholesterol synthesis per unit area of SER membranes in Leydig cells. As mentioned above, a majority of the 30 or more enzymes of cholesterol synthesis are located on the SER of Leydig cells (8). Morris and Chaikoff (22) have estimated that ~60% of the cholesterol in the rat testis is synthesized *in situ*, while 40% is derived from the plasma. Let us assume that the *in situ* synthesis takes place predominantly in the Leydig cells, and that each molecule of cholesterol is converted into a molecule of testosterone, which is not unreasonable as rat Leydig cells have very few lipid droplets and would thus store little of the cholesterol they synthesize (see reference 8). We can then estimate that each square centimeter of SER produces ~3.4 ng of cholesterol/d, or, in other words, 61 million molecules/s. The cholesterol would begin its conversion to testosterone by cleavage of the sidechain to produce pregnenolone. This sidechain cleavage takes place on the inner mitochondrial membrane, where the cleavage enzymes on each square centimeter of membrane would convert ~12.0 ng of cholesterol/d to pregnenolone.

The functional activity of Leydig cells is regulated by luteinizing hormone (LH), which binds to LH receptors on the Leydig plasma membrane (6). Conn et al. (11) estimate that there are ~20,000 LH receptors/Leydig cell. Assuming that these receptors are randomly distributed over the cell surface, then the results of the present paper would suggest that there are ~13 LH receptors/μm^2 of plasma membrane.

The comparative abundance of SER membranes in Leydig cells varies from species to species (see references 8 and 9 for reviews). Morphometric studies on the Leydig cells of guinea pigs, mice, and other species are underway to furnish a better quantitative understanding of this cell type in various mammals. The results of these and other studies, coupled with biochemical and physiological information, may make it possible to characterize the detailed internal economy of Leydig cells in quantitative terms. We hope that the present paper has furnished some progress toward this general goal, a quantitative cell biology of Leydig cells.

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