STEREOLOGICAL ANALYSIS OF HEPATIC FINE STRUCTURE IN THE FISCHER 344 RAT

Influence of Sublobular Location and Animal Age

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ABSTRACT

Stereological analysis of hepatic fine structure in Fischer 344 male rats at 1, 6, 10, 16, 20, 25, and 30 mo of age revealed differences in the amounts and distributions of hepatocellular organelles as a function of sublobular location or animal age. Between 1 and 16 mo of age, both the centrolobular and periportal hepatocytes increased in volume by 65 and 35%, respectively. Subsequently, the cell volumes declined until the hepatocytes of 30-mo-old rats approached the size of those found in the youngest animals. Regardless of animal age, the centrolobular cells were consistently larger than the corresponding periportal hepatocytes. The cytoplasmic and ground substance compartments reflected similar changes in their volumes, although there was no significant alteration in the nuclear volume.

The volumes of the mitochondrial and microbody compartments increased and decreased concomitant with the changes in average hepatocyte size. Both lobular zones in the 30-mo-old rats contained significantly smaller relative volumes of mitochondria than similar parenchyma in 16-mo-old animals. The volume density of the dense bodies (lysosomes) increased markedly in both lobular zones between 1 and 30 mo of age, confirming reports of an age-dependent increase in this organelle. The surface area of the endoplasmic reticulum in the centrolobular and periportal hepatocytes reached its maximum level in the 10-mo-old rats and subsequently declined to amounts which approximated those measured in the 1-mo-old animals. This age-related loss of intracellular membrane is attributable to a significant reduction in the surface area of the smooth-surfaced endoplasmic reticulum (SER) in animals beyond 16 mo of age. The amount of rough-surfaced endoplasmic reticulum (RER) in the periportal parenchymal cells was unaffected by aging, but the centrolobular hepatocytes of 30-mo-old animals contained 90% more RER than similar cells in the youngest rats. The centrolobular parenchyma contained more SER and the portal zones more RER throughout the age span studied. These quantitative data suggest that (*a*) certain hepatic fine structural parameters undergo marked changes as a function of animal age, (*b*) there exists a gradient in hepatocellular fine structure across the entire liver lobule, and (*c*)

there are remarkable similarities in hepatocyte ultrastructure between very young and senescent animals, including cell size and the amount of SER.

KEY WORDS liver structure · aging · stereology · hepatocyte heterogeneity · endoplasmic reticulum

Several studies reported a heterogeneity in the lobular distribution of a number of hepatic functions, including glycogenesis and glycogenolysis (5, 8, 22, 38), albumin synthesis (26), drug metabolism (7), as well as in the sublobular localization of certain enzyme activities (29, 30, 54). Furthermore, the classic stereological study of Loud (27) demonstrated differences in the amounts or distributions of several hepatocyte organelles as a function of the sublobular location of the parenchymal cells. Finally, several investigators successfully isolated two apparently distinct fractions of rat hepatocytes by density gradient centrifugation and reported differences in cell size, cell density, the amount of smooth-surfaced endoplasmic reticulum (SER) and the configuration of the glycogen deposits (9, 14, 51). These two classes of hepatocytes were equated to centrolobular and periportal cells, although which group corresponds to which lobular zone remains unclear (14, 51).

Loud (27) evaluated the hepatocytes comprising a 1–4-cell radius around the central vein (terminal hepatic venule) and the portal triad, as well as a narrow midlobular zone. This method included those cells comprising the limiting plates and maximized any sublobular differences in hepatocyte fine structure. Furthermore, this approach did not clearly demonstrate whether a gradient of differences extended across the entire radius of the liver lobule. As a result, Loud (27) suggested that 80% of the liver lobule consisted of a homogeneous population of hepatocytes, primarily midlobular cells. If this is true, only those cells immediately surrounding the terminal afferent and efferent blood vessels exhibit significantly different fine-structural qualities, whereas the midlobular hepatocytes possess intermediate characteristics.

However, a recent stereological analysis of the rat liver demonstrated quantitative differences in hepatocyte ultrastructure across most of the liver lobule (20). Since these investigators attempted to exclude the 2–3-cell layer immediately surrounding either the central vein or portal triad, the differences in cell fine structure between the two extremes of the lobule probably cannot account for the significant differences observed between the remaining central and portal portions. The concept of a lobular gradient in hepatocyte fine structure is consistent with the current data describing a functional heterogeneity across the liver lobule.

Most stereological analyses of hepatocyte ultrastructure employed young rats, usually 3 mo of age or less, and did not consider the influence of sublobular location. Recent studies demonstrated that the aging process affects certain hepatic fine-structural parameters in the rat (32, 33, 41–44, 24, 50, 57). Aging is responsible for a number of distinct changes in hepatic function, including decreased drug metabolism (11), reduced turnover and excretion of cholesterol (18, 25), and latent adaptive responsiveness (1, 2). However, there have been few attempts to correlate such age-dependent changes in liver function with alterations in hepatocyte fine structure.

Age-related changes in cell structure and function may go unnoticed since most aging studies evaluate only two age points, usually comparing young vs. senescent animals. The present study was designed to quantitate and compare hepatic fine structure at seven specific age intervals throughout the life-span of a carefully controlled colony of aging rats, including periods of development, maturation, and senescence. A quantitative description of liver cell fine structure as a function of animal age and sublobular location is important to develop (*a*) potential model systems for the study of aging in the liver, such as hepatocytes in primary monolayer culture, and (*b*) more physiological methods for isolating hepatocytes from different sublobular zones, i.e., alternatives to employing phenobarbital-induced SER proliferation to alter their buoyant density (51) or selectively destroying centrolobular or periportal cells with bromobenzene or allyl alcohol.

MATERIALS AND METHODS

Animals

Male Fischer 344 (CDF) rats obtained from the aging colony maintained at the Charles River Breeding Laboratories, Wilmington, Mass., under contract to the National Institute on Aging, were used throughout the study. This particular colony of rats is barrier-bred and

-sustained, carefully monitored, and used solely for aging studies. The incidence and severity of a wide variety of age-related pathological conditions have been evaluated in these animals (10).

Groups of six animals each at the ages of 1, 6, 10, 16, 20, 25, and 30 mo (100, 100, 100, 100, 96, 77, and 26% survivorship, respectively) were received 1 wk before use, maintained in an isolated room in separate cages and fed Purina Laboratory Chow (Ralston Purina Co., St. Louis, Mo.) and water ad lib.

Perfusion Fixation

The animals were fasted for 18 h before sacrifice to minimize dietary variations and to avoid heavy accumulations of glycogen in the hepatocytes. Anesthesia was induced by the intraperitoneal administration of sodium pentobarbital (5 mg/100 gm body weight). The livers were perfused via the hepatic portal vein (58) with 2.7% glutaraldehyde-0.8% paraformaldehyde in 0.2 M sodium bicarbonate buffer (pH 7.4; 770 mosmol). After *in situ* perfusion, tissue from the median lobe was removed, cut into small pieces, and immersed in the same fixative for an additional 2 h at room temperature. The tissue was rinsed in buffer overnight, postfixed in 1% osmium tetroxide-1.5% potassium ferrocyanide, dehydrated in ethanol, and embedded in Epon (28). Neither liver weights nor volumes were determined because the study was primarily concerned with relative, i.e., volume and surface densities, rather than absolute estimates of cell and organelle volumes and surface areas.

Perfusion of the liver with a hypertonic fixative may complicate the interpretation of fine-structural data as well as the comparison of these results with those obtained in other studies employing immersion fixation and/or isotonic fixatives. However, Pilström and Nordlund (34) and Blouin et al. (6) reported definite advantages to using hypertonic fixatives and perfusion fixation for the preservation of rat liver tissue for stereological studies.

Thick sections (~0.5 μm) were stained with toluidine blue and used to determine the lobular orientation of each tissue block. Six tissue blocks, three containing central veins and three containing portal triads in cross section, were selected from each of the six animals per age group. These randomly chosen blocks were retrimmed to within a 10-cell radius of the central vein or portal triad. According to the data of Loud (27), these sample areas contained midlobular as well as centrolobular or periportal hepatocytes. A previous analysis of thick sections containing both central veins and portal triads revealed that the average rat liver lobule had a radius of 24 hepatocytes (42). This sampling procedure effectively eliminated the midlobular cells by incorporating them into the arbitrarily defined centrolobular and portal zones. Thin sections of silver to grey interference colors (~50–60 nm) were cut from the selected tissue blocks and doubly stained with uranyl acetate and lead citrate.

Sampling Procedure

Three different magnification levels were required for sampling. Two light micrographs, each of central and portal zone parenchyma, at a final magnification of 1,000, were selected for every animal and used to estimate the volume densities of the hepatocytes and the extrahepatocytic space, average hepatocyte volumes, and nuclear numerical densities according to the procedure described by Loud (27). A square single lattice test system corresponding to an area of 10,000 μm^2 of intralobular parenchyma was used for point counting.

Five electron micrographs per tissue block were taken at each of two primary magnifications, 4,600 and 15,200, and photographically enlarged to 13,800 and 45,600, respectively. The photographic fields were selected according to an unbiased, systematic sampling procedure which required that (*a*) the grid squares be totally covered with intact tissue and (*b*) two sides of the photographic field be defined by grid bars (20, 41). An effort was made to avoid photographing those hepatocytes comprising the 2–3-cell layer immediately surrounding either a central vein or portal triad. This yielded 90 low- and 90 high-magnification electron micrographs per central and portal zone, respectively, for each age group. A total of 2,520 electron micrographs was evaluated during the course of this study.

The volume densities of the hepatocyte cytoplasmic and ground substance compartments, the nuclei, mitochondria, microbodies (peroxisomes), dense bodies (lysosomes), cytoplasmic lipid droplets, Golgi-rich areas (19, 20), and bile canalicular space were estimated on the low-magnification electron micrographs. A coherent double lattice test system (1:16 coarse to fine points) was employed for this purpose according to the point-counting procedure described by Weibel et al. (52) using the intralobular parenchyma as the reference volume.

Only the canalicular space and not the microvilli projecting into the lumina was considered in the volume estimates of the biliary space. All of the lysosomes were grouped as dense bodies because of their multiplicity of forms, ranging from primary lysosomes to residual lipofuscin bodies. The reliability of estimating the surface area of the Golgi membranes on the high-magnification electron micrographs was questionable since the relative paucity of this organelle resulted in a low incidence of membrane profiles. As an additional quantitative method, the Golgi complex was treated as a specific region, i.e., the extreme boundaries of each Golgi complex were outlined on every low-magnification electron micrograph, and these regions were arbitrarily defined as "Golgi-rich areas" (19, 20). These zones contained the lamellar saccules of the Golgi complex, any associated large or small vesicles, and the intervening ground substance. The fine points of the coherent

double lattice test system which fell within the Golgi-rich areas were used to estimate the volume density of this organelle.

Surface densities of rough- (RER) and smooth-surfaced endoplasmic reticulum and Golgi membranes were estimated on the high-magnification electron micrographs using a coherent multipurpose test system similar to that described by Weibel et al. (52). The hepatocytic ground substance compartment, which included the RER, SER, Golgi complex, and cytoplasmic lipid droplets, served as the reference volume for all estimates of membrane surface density. This mode of expression of data was used since the microsomes (RER, SER, Golgi complex) are usually the last fraction isolated during subcellular fractionation procedures, leaving only the ground substance or 105,000-*g* supernate. Data on membrane surface areas expressed in this manner can be more easily correlated with biochemical results based on the percent recovery of microsomes.

The close associations and anastomoses between profiles of the RER and SER suggested that a very critical method was necessary to categorize these respective membrane systems and to avoid errors introduced during the counting procedure. The tubular configurations of smooth membrane, as well as those portions of parallel lamellae devoid of ribosomes for lengths in excess of 200 nm, were counted as SER.[1]

The volume data are expressed as (*a*) volume densities or geometrical units, i.e., cubic centimeters per cubic centimeter of intralobular liver tissue, and (*b*) biological units or volume/average mononuclear hepatocyte (cubic micrometers per cell) (27). The surface areas of the intracellular membranes are expressed as surface densities (square meters per cubic centimeter of hepatocyte ground substance) and as the surface area per average mononuclear parenchymal cell.

The expression of stereological data as specific values, i.e., per average mononuclear hepatocyte (27), assumes either that binucleate parenchymal cells are approximately twice the size of mononuclear cells or that the number of binucleate hepatocytes relative to mononucleate parenchymal cells remains reasonably constant as a function of animal age. To determine the validity of this mode of expression of data, a total of 3,600 centrolobular and portal hepatocytes in 1-, 16-, and 30-mo-old rats were classified according to mono- or binuclear profiles.[2] There was no significant difference in the binucleate/mononucleate hepatocyte ratio as a function of either sublobular location or animal age. Furthermore, Elias (15) reported that binucleate rat hepatocytes are twice as large as mononucleate parenchymal cells and Doljanski (13) demonstrated that the hepatocyte nucleocytoplasmic ratio remains constant as a function of age in the rat. These data suggest that the volume of cytoplasm associated with each parenchymal cell nucleus probably approximates the actual volume of an average mononuclear hepatocyte (27).

The confidence level of sampling for all estimates of volume density exceeded 95% according to the equation described by Weibel (53). The point or intersection counts obtained from each tissue block from the low- and high-magnification electron micrographs, respectively, were pooled and represented a sample unit. All of the stereological data were subjected to a statistical computation, including a probability analysis (Student's *t* test).

[1] Recent reports described several systematic errors in the application of point-counting stereology which may result in either over- or underestimations of organelle volume and/or surface areas (6, 53). Blouin et al. (6) found a 17% reduction in the dimensions of tissue sections due to compression during sectioning. Although we cannot comment on the degree of hardness of the embedding medium or the sharpness of the knives employed by these investigators (6), we assume that all of the tissue sections examined in the present study were subjected to minimal compression forces and consider this potential error to be relative throughout the entire sample population. Considerable care was exercised during tissue sectioning to obtain sections of uniform thickness based on interference colors. Although this procedure is not so exact as that described by Small (40), we suggest that section thickness was reasonably constant and that any errors in the estimates of organelle volumes resulting from the Holmes effect are evenly distributed throughout the samples. Furthermore, no attempt was made to correct for possible errors in volume or surface estimates which may have resulted from the angle of section, variations in organelle profiles, or specific membrane configurations.

RESULTS

Preservation of Hepatic Fine Structure and Qualitative Observations

In situ perfusion fixation resulted in excellent preservation of hepatic fine structure (Figs. 1 and 2). The sinusoidal lumina were devoid of erythro-

[2] Several problems are inherent in attempts to extrapolate three-dimensional data from two-dimensional images, as in stereological studies. However, the application of "correction factors", such as Wheatley's (55), complicates matters by attempting to obtain absolute values from estimates. Our purpose in determining the distribution of mono- and binucleate hepatocytes as a function of age is to demonstrate the relative frequency of these cell types rather than to compare specific changes in the number of binucleated parenchymal cells. The livers of these particular animals never contain >10% binucleated hepatocytes regardless of age. Thus, the impact of this factor on the overall data must be considered minimal.

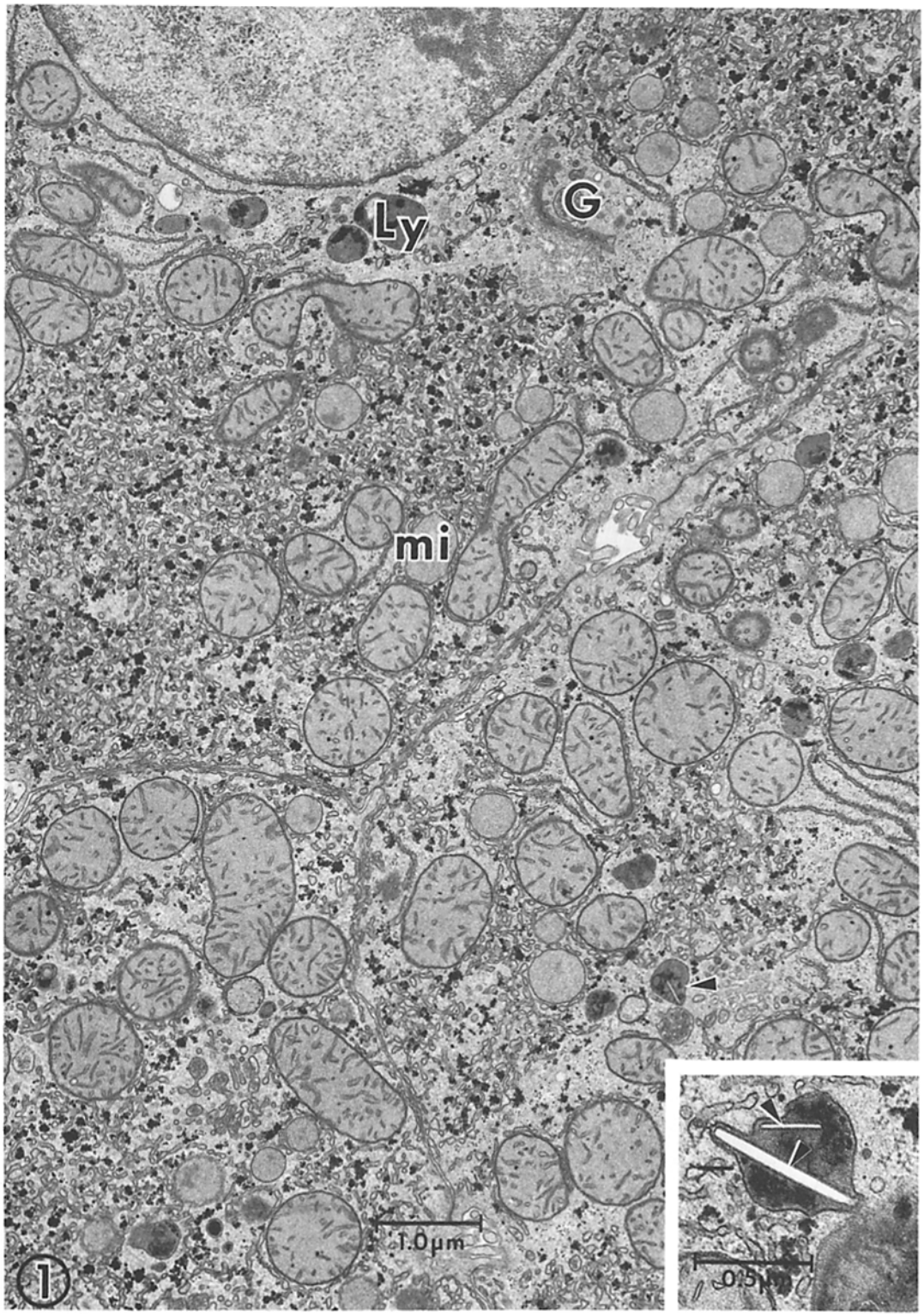

FIGURE 1 Electron micrograph of perfusion-fixed centrolobular liver tissue from a 16-mo-old rat illustrating typical hepatocyte fine structure. The lysosomes or dense bodies (*Ly*) contained a moderate amount of electron-opaque material, presumably lipofuscin, and sometimes crystalloid inclusions (arrowhead). *G*, Golgi complex; *mi*, microbodies. Inset: high magnification of a dense body containing crystalloid inclusions (arrowheads). ×13,800; bar, 1 μm. Inset, ×33,350; bar, 0.5 μm.

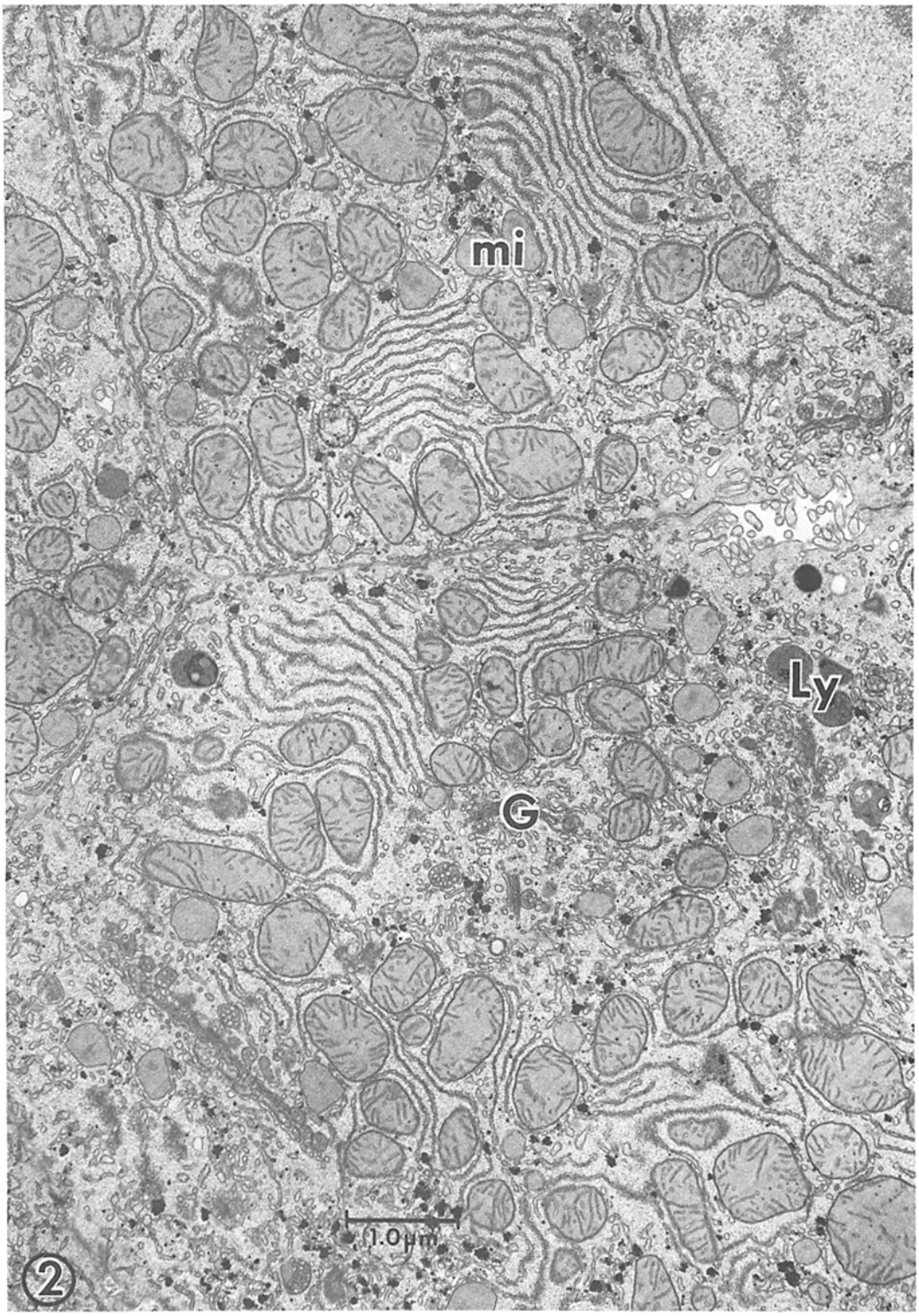

FIGURE 2 Portions of several portal zone hepatocytes from a 30-mo-old rat after perfusion fixation. No obvious differences in liver cell fine structure attributable to age or lobular location were detected during qualitative examination. *Ly,* dense bodies; *mi,* microbodies; *G,* Golgi complex. ×13,800; bar, 1 μm.

cytes, the endothelial and Kupffer cells remained intact, and the space of Disse was easily observed. There was no evidence of injury attributable to excessive perfusion pressure or flow rate. No attempt was made to quantitate changes in the fine structure of the nonhepatocytic elements, i.e., endothelial cells, Kupffer cells, and fat-storing cells.

Most hepatocyte organelles appeared to be evenly distributed throughout the cytoplasm, except the dense bodies and the Golgi complexes, both of which were preferentially oriented toward the bile canalicular surfaces of the liver cells. The only apparent differences in hepatocyte ultrastructure were: (*a*) a greater number of cytoplasmic lipid droplets in the portal cells in comparison to the centrolobular hepatocytes and (*b*) an age-dependent increase in the number, size, and lipofuscin or lipid content of the dense bodies (Fig. 3). Numerous dense bodies containing crystalloid

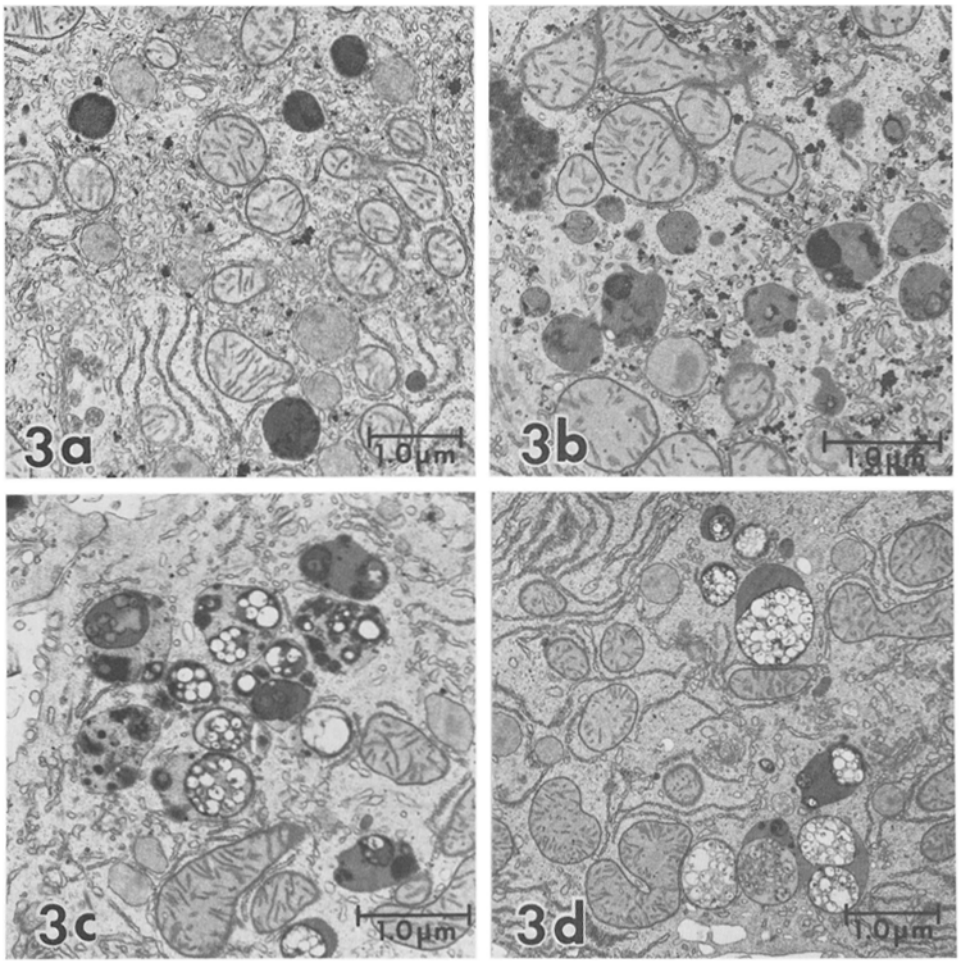

FIGURE 3 Electron micrographs demonstrating the age-related changes in the appearance of dense bodies. (*a*) Hepatocyte dense bodies in 1-mo-old rats present a typical appearance with little or no lipofuscin in their matrices. ×10,000; bar, 1 μm. (*b*) Dense bodies in the livers of 16-mo-old rats contained a moderate amount of lipofuscin. ×15,000; bar, 1 μm. (*c*) The matrices of dense bodies in hepatocytes of 25-mo-old animals were largely composed of lipofuscin. However, aggregates of electron-transparent material, presumably lipoidal, have begun to replace the lipofuscin. ×15,000; bar, 1 μm. (*d*) By 30 mo of age the dense bodies were considerably larger and contained great amounts of lipoidal material. In some of these organelles the lipofuscin was polarized by the accumulating amounts of lipoidal material. ×10,000; bar, 1 μm.

inclusions were found in hepatocytes regardless of animal age or sublobular location (Fig. 1, inset). These particular organelles were often found in close association with more typical lysosomes. Lastly, the body weight of these rats increased steadily through 20 mo of age, and the only statistically significant weight loss occurred between 25 and 30 mo of age (20%; $P < 0.01$).

Quantitative Analysis of Hepatocyte Fine Structure

Differences in the volumes of hepatocytes and their constituent ground substance compartments were of interest because much of the stereological data was expressed relative to both of these reference units. The volume density of the parenchymal cells did not change appreciably with animal age (Fig. 4, top). The only significant differences were: (*a*) an 8% increase in the volume density of portal hepatocytes between 1 and 6 mo of age ($P < 0.05$; Table I) and (*b*) a larger hepatocyte volume density in the centrolobular parenchyma in comparison to portal tissue in 1-mo-old rats ($P < 0.05$; Table I). However, the volume of individual hepatocytes in both zones increased between 1 and 16 mo and subsequently declined until, in the 30-mo-old animals, cell sizes approached those found in the youngest age group (Fig. 4, bottom). There was no significant difference in the volume of periportal hepatocytes between 1 and 30 mo of age. The centrolobular parenchymal cells were consistently larger than the portal cells throughout the life-span, although these differences were significant only in the 10-, 16-, 20-, and 30-month age groups ($P < 0.05 - 0.01$; Table I).

The changes in the volume densities and specific volumes of hepatocyte cytoplasmic and ground substance compartments reflected those observed in the parenchymal cells, except that the volume density of the ground substance increased markedly between 25 and 30 mo of age (Table I). At all age intervals studied, the centrolobular hepatocytes contained larger volumes of cytoplasm and ground substance than the portal cells. The volume density of the hepatocyte nuclei fluctuated considerably throughout the life-span, but there was no consistent difference in this parameter between the two sublobular zones or between the youngest and oldest age groups (Table I). In general, the specific volumes of the nuclei reflected the minor changes in volume density, although these differences were significant between several age groups. The centrolobular hepatocytes of 16-, 20-, and 30-mo-old rats contained larger nuclei than corresponding portal cells, and the nuclei of centrolobular cells of the 30-mo-old animals were significantly larger than those in the 1-mo-old rats ($P < 0.05$). The nuclear numerical density (number of hepatocyte nuclei per volume of intralobular parenchyma) decreased between 1 and 10 mo of age in both lobular zones and subsequently returned to the initial level in the portal tissue. However, there was a net loss in this parameter in the centrolobular tissue between 1 and 30 mo of age (Table I).

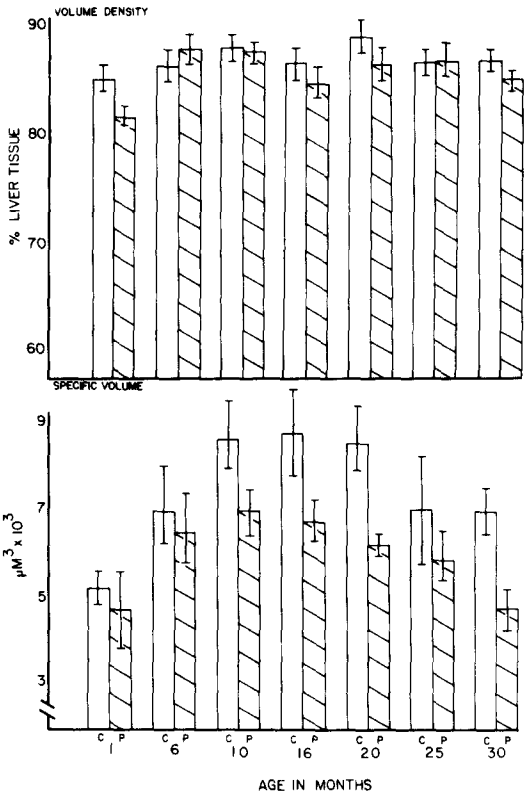

FIGURE 4 The effects of animal age and sublobular location on hepatocyte volume are demonstrated in this graph. The top graph represents the age-related changes in the volume densities of centrolobular (hatched bars) and portal (clear bars) zone hepatocytes. There are no major changes in hepatocyte volume density in either lobular zone. The average volume of individual hepatocytes is represented in the bottom graph. The centrolobular cells are consistently larger than the portal cells within each age group. There is a significant increase in average cell volume in both zones between 1 and 16 mo, followed by a significant decline, at least in the portal hepatocytes, between 16 and 30 mo.

TABLE I

Effects of Aging and Sublobular Location on Hepatic Fine-Structural Parameters in Fischer 344 Male Rats

COMPONENTS *	1 MONTH		6 MONTHS		10 MONTHS		16 MONTHS		20 MONTHS		25 MONTHS		30 MONTHS	
	CENTRAL	PORTAL	CENTRAL	PORTAL	CENTRAL	PORTAL	CENTRAL	PORTAL	CENTRAL	PORTAL	CENTRAL	PORTAL	CENTRAL	PORTAL
EXTRAHEPATOCYTE ‡	.15 ±.01	.18 ±.01	.14 ±.02	.12 ±.02	.12 ±.01	.12 ±.01	.13 ±.02	.15 ±.02	.10 ±.01	.14 ±.02	.13 ±.01	.13 ±.02	.13 ±.01	.15 ±.01
BILIARY SPACE	.008 ±.001	.006 ±.001	.007 ±.001	.008 ±.002	.006 ±.001	.017 ±.003	.011 ±.002	.019 ±.002	.014 ±.003	.022 ±.003	.008 ±.001	.017 ±.003	.011 ±.002	.022 ±.003
HEPATOCYTE	.85 ±.01	.82 ±.01	.86 ±.02	.88 ±.02	.88 ±.01	.88 ±.01	.87 ±.02	.85 ±.02	.89 ±.01	.86 ±.02	.87 ±.01	.87 ±.02	.87 ±.01	.85 ±.01
§	*5242 ±414*	*4742 ±899*	*7050 ±971*	*6532 ±884*	*8614 ±895*	*7032 ±539*	*8784 ±1012*	*6756 ±447*	*8563 ±829*	*6226 ±274*	*7064 ±1316*	*5895 ±732*	*6998 ±564*	*4787 ±508*
NUCLEUS	.07 ±.01	.07 ±.01	.07 ±.01	.08 ±.01	.06 ±.01	.06 ±.01	.06 ±.01	.05 ±.01	.09 ±.01	.07 ±.01	.06 ±.01	.09 ±.01	.08 ±.01	.07 ±.01
	449 ±43	*378 ±47*	*547 ±57*	*565 ±89*	*548 ±98*	*497 ±72*	*629 ±71*	*406 ±72*	*818 ±77*	*504 ±77*	*464 ±65*	*597 ±68*	*678 ±65*	*383 ±51*
‖	165 ±14	159 ±9	126 ±14	133 ±16	104 ±8	125 ±12	104 ±12	126 ±6	104 ±9	141 ±8	138 ±18	154 ±22	119 ±11	166 ±17
CYTOPLASM	.78 ±.01	.75 ±.01	.79 ±.02	.80 ±.02	.82 ±.01	.82 ±.01	.80 ±.02	.80 ±.01	.81 ±.01	.79 ±.02	.81 ±.02	.78 ±.02	.78 ±.01	.78 ±.01
	4793 ±80	*4364 ±64*	*6462 ±172*	*5967 ±134*	*8066 ±137*	*6535 ±88*	*8155 ±162*	*6397 ±112*	*7755 ±125*	*5722 ±115*	*6632 ±130*	*5298 ±129*	*6320 ±113*	*4404 ±68*
GROUND SUBSTANCE	.59 ±.01	.55 ±.01	.61 ±.01	.60 ±.01	.65 ±.01	.56 ±.01	.59 ±.01	.56 ±.01	.57 ±.01	.55 ±.01	.56 ±.01	.55 ±.01	.62 ±.01	.60 ±.01
	3618 ±62	*3185 ±52*	*4975 ±82*	*4464 ±82*	*6333 ±117*	*4450 ±64*	*5974 ±101*	*4469 ±72*	*5455 ±96*	*3963 ±65*	*4571 ±81*	*3711 ±68*	*4988 ±97*	*3402 ±51*
CYTOPLASMIC LIPID	.003 ±.002	.007 ±.002	.006 ±.001	.015 ±.005	.007 ±.002	.011 ±.003	.017 ±.004	.019 ±.003	.010 ±.002	.015 ±.003	.009 ±.003	.021 ±.004	.005 ±.001	.010 ±.004
	18 ±12	*41 ±12*	*49 ±8*	*112 ±37*	*69 ±20*	*88 ±24*	*172 ±40*	*151 ±24*	*96 ±19*	*108 ±22*	*73 ±24*	*142 ±27*	*40 ±8*	*56 ±22*

Values represent the Mean ±standard error of the mean

* Includes intralobular liver tissue only

‡ Initial values for each component are expressed as volume densities, i.e. cm^3/cm^3 of intralobular liver tissue.

§ Italicized values are expressed as specific volumes, i.e. μM^3/average mononuclear hepatocyte.

‖ Nuclear numerical density or the number of hepatocyte nuclei/cm^3 of intralobular liver tissue. ($\times 10^6$).

The volume of bile canalicular space increased markedly in the portal (113%) and centrolobular (133%) parenchyma between 6 and 20 mo of age (Table I). There were no age-related or lobular differences in biliary space between 1 and 6 mo, although in animals beyond 6 mo of age the portal tissue contained significantly larger volumes of biliary space than the centrolobular parenchyma ($P < 0.01 - 0.001$). Cytoplasmic lipid droplets, presumably representing triglycerides, increased in both sublobular zones between 1 and 16 mo and returned to the initial levels by 30 mo (Table I). The relative and specific volumes of lipid droplets were consistently greater in the portal parenchyma in all age groups.

Mitochondrial volume density in the portal tissue increased by 23% between 6 and 10 mo and subsequently declined until the 30-mo-old rats contained significantly less mitochondrial volume than the youngest animals ($P < 0.05$; Table II). This stereological parameter also increased in the centrolobular parenchyma (42%) until the animals reached 25 mo of age and then decreased, resulting in a net loss by 30 mo ($P < 0.05$). In general, the portal parenchyma contained a larger volume of mitochondria than the centrolobular tissue. The specific volumes of mitochondria exhibited a similar age-related pattern, although there was no difference in either zone between the youngest and oldest rats (Table II). Contrary to the volume density data, the centrolobular hepatocytes in animals beyond 10 mo of age contained significantly larger volumes of mitochondria than their respective portal zone cells ($P < 0.05$).

The age-dependent distribution patterns of microbodies were similar to those found for the mitochondria, except that there were no net differences between the 1- and 30-mo-old animals (Table II). The lobular distributions varied, but the centrolobular parenchymal cells in the older rats contained greater volumes of microbodies than the portal hepatocytes. However, the volume density of dense bodies increased significantly in the centrolobular and periportal parenchyma, 150% and 175%, respectively, between 6 and 30 mo of age (Table II). Although the differences were not statistically significant, the portal tissue generally contained a larger volume of dense bodies than the central zone parenchyma. The volume of dense bodies per individual hepatocytes also increased in the centrolobular (81%) and portal (122%) cells as a function of age ($P < 0.01$).

The surface density of the total endoplasmic reticulum (ER), comprised of the SER and RER, increased in the centrolobular (34%) and portal (20%) zones between 1 and 6 mo, but subsequently decreased until the levels measured in the 30-mo-old animals were 13 and 19% below those found in the youngest rats (Fig. 5; Table II). This apparent age-dependent loss of membrane reflected a concomitant reduction in SER surface density in the central (15%) and portal (21%) tissue. The RER surface density did not change appreciably throughout the life-span, and the liver tissue of the youngest and oldest animals contained similar amounts of this membrane (Fig. 5; Table II). A definite lobular gradient was noted in the distribution of membrane surface densities wherein the centrolobular parenchyma consistently contained more SER and the portal tissue contained slightly more RER.

Differences in the amounts or distributions of ER were more obvious when these data were expressed per individual hepatocyte. The total ER in centrolobular cells more than doubled in surface area between 1 and 10 mo of age ($P < 0.001$; Fig. 6; Table II), although this age-related increase was less marked in the portal hepatocytes (65%; $P < 0.001$). Beyond 10 mo of age the surface area of the ER declined by 42% in the centrolobular cells and by 45% in the periportal hepatocytes. Thus, the centrolobular parenchymal cells in the oldest rats contained significantly more ER than similar cells in the 1-mo-old animals ($P < 0.01$). However, the age-dependent loss of ER in the portal cells eliminated any significant difference between the youngest and oldest rats. With the exception of the 1-mo age group, the centrolobular hepatocytes consistently contained significantly more ER than the periportal cells in rats at any given age.

The estimates of RER and SER surface areas per average parenchymal cell confirmed that the age-related loss of ER was attributable to a reduction in the amount of SER (Fig. 7; Table II). The portal hepatocytes in the 30-mo-old animals contained ~16% less SER than those in the 1-mo-old rats. Although the central zone cells contained more SER than the portal hepatocytes throughout the entire age span studied, the suspected lobular gradient in the distribution of RER was not confirmed by the specific surface area data (Fig. 7). The amount of RER in individual centrolobular hepatocytes increased significantly between 1 and 10 mo of age (127%; $P < 0.001$). The

TABLE II

Effects of Aging and Sublobular Location on Hepatic Fine-Structural Parameters in Fischer 344 Male Rats

COMPONENTS *	1 MONTH		6 MONTHS		10 MONTHS		16 MONTHS		20 MONTHS		25 MONTHS		30 MONTHS	
	CENTRAL	PORTAL	CENTRAL	PORTAL	CENTRAL	PORTAL	CENTRAL	PURTAL	CENTRAL	PORTAL	CENTRAL	PORTAL	CENTRAL	PORTAL
MITOCHONDRIA ‡	.17 ±.01	.19 ±.01	.17 ±.01	.18 ±.01	.16 ±.01	.23 ±.01	.19 ±.01	.21 ±.01	.21 ±.01	.21 ±.01	.23 ±.01	.20 ±.01	.15 ±.01	.16 ±.01
	1052 ±49	*1075 ±70*	*1356 ±57*	*1369 ±52*	*1566 ±108*	*1804 ±72*	*1907 ±61*	*1673 ±56*	*1992 ±96*	*1528 ±58*	*1850 ±54*	*1384 ±81*	*1178 ±48*	*879 ±39*
MICROBODIES	.015 ±.001	.014 ±.001	.012 ±.001	.014 ±.001	.013 ±.001	.024 ±.001	.019 ±.001	.022 ±.001	.022 ±.001	.021 ±.001	.017 ±.001	.019 ±.001	.012 ±.001	.013 ±.001
	92 ±6	*81 ±6*	*98 ±8*	*104 ±7*	*127 ±10*	*192 ±8*	*193 ±10*	*175 ±8*	*212 ±10*	*151 ±7*	*139 ±8*	*129 ±7*	*97 ±8*	*73 ±6*
DENSE BODIES	.005 ±.001	.004 ±.000	.004 ±.000	.004 ±.001	.004 ±.000	.011 ±.001	.008 ±.001	.010 ±.001	.010 ±.001	.011 ±.001	.009 ±.001	.011 ±.002	.007 ±.001	.009 ±.001
	31 ±6	*23 ±2*	*33 ±0*	*30 ±8*	*39 ±4*	*88 ±8*	*81 ±10*	*80 ±8*	*96 ±10*	*79 ±7*	*73 ±8*	*75 ±14*	*57 ±8*	*51 ±6*
ROUGH SURFACED § ENDOPLASMIC RETICULUM	4.32 ±.35	4.57 ±.32	4.61 ±.44	5.27 ±.43	3.86 ±.29	4.23 ±.37	3.45 ±.31	4.45 ±.33	3.00 ±.32	3.78 ±.33	3.24 ±.26	4.69 ±.34	4.11 ±.24	4.27 ±.22
	15628±1274	*14563 ±1020*	*22945 ±2190*	*23511 ±1918*	*24439 ±1836*	*18799 ±1644*	*20607 ±1852*	*19872 ±1474*	*16364 ±1745*	*14968 ±1307*	*14808 ±1188*	*17390 ±1261*	*20478 ±1196*	*14513 ±748*
SMOOTH SURFACED ENDOPLASMIC RETICULUM	8.10 ±.32	7.93 ±.32	12.01 ±.82	9.77 ±.48	11.00 ±.56	10.55 ±.56	11.75 ±.65	9.46 ±.46	11.00 ±.57	8.84 ±.46	8.55 ±.61	6.42 ±.35	6.87 ±.29	6.27 ±.20
	29298 ±1157	*25270 ±1020*	*59777 ±4081*	*43587 ±2141*	*69644 ±3546*	*46887 ±2489*	*70184 ±3883*	*42246 ±2054*	*60000 ±3109*	*35004 ±1821*	*39077 ±2788*	*23805 ±1298*	*34230 ±1445*	*21310 ±680*
GOLGI	.46 ±.09	.49 ±.11	.49 ±.12	.86 ±.30	.58 ±.15	.72 ±.10	.52 ±.14	.61 ±.13	.52 ±.10	.69 ±.10	.73 ±.10	.85 ±.13	.38 ±.11	.77 ±.14
	1664 ±326	*1561 ±350*	*2439 ±597*	*3837 ±1338*	*3672 ±950*	*3200 ±444*	*3106 ±836*	*2724 ±581*	*2836 ±545*	*2732 ±396*	*3336 ±457*	*3152 ±482*	*1893 ±548*	*2617 ±476*
GOLGI-RICH AREA ‖	.017 ±.001	.016 ±.001	.010 ±.001	.013 ±.002	.011 ±.001	.015 ±.001	.013 ±.001	.015 ±.001	.012 ±.002	.013 ±.001	.013 ±.001	.013 ±.001	.014 ±.001	.018 ±.001
	105 ±6	*93 ±6*	*82 ±8*	*97 ±15*	*108 ±10*	*120 ±8*	*132 ±10*	*120 ±8*	*115 ±19*	*94 ±7*	*106 ±8*	*88 ±7*	*113 ±8*	*101 ±6*

Values represent the Mean ± standard error of the mean

* Includes intralobular tissue only

‡ Volume estimates are expressed as volume densities (cm^3/cm^3 of intralobular liver tissue) and specific volumes (μM^3/average mononuclear hepatocyte; italicized values).

§ Estimates of membrane surface areas are expressed as surface densities (M^2/cm^3 of hepatocytic ground substance) and specific surface areas (μM^2/average mononuclear hepatocyte; italicized values).

‖ Arbitrarily defined region consisting of the Golgi saccules, large and small vesicles and the intervening ground substance.

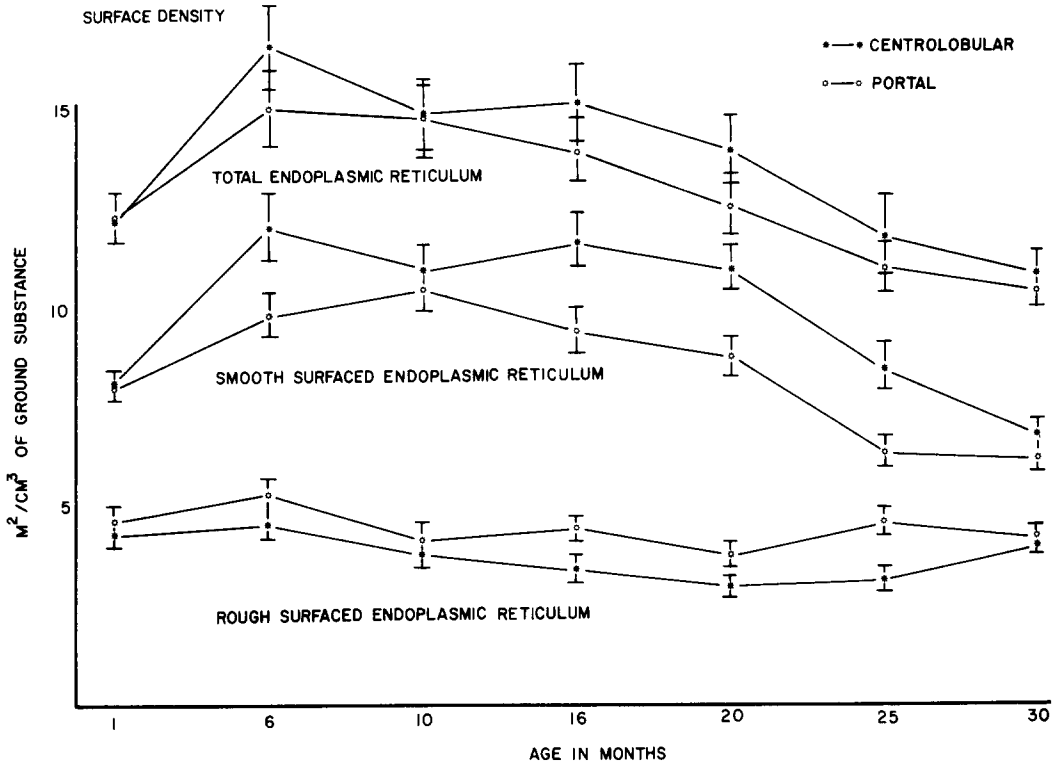

FIGURE 5 This graph demonstrates the changes in the surface densities (surface to volume ratio) of the total, smooth, and rough endoplasmic reticulum membranes as a function of animal age and lobular location of hepatocytes. After an initial increase in total ER between 1 and 6 mo, the surface density declines steadily until the hepatic parenchyma in 30-mo-old rats contains less membrane than that in 1-mo-old animals. The SER follows a similar age-related pattern, whereas there is no net change in RER surface density. The centrolobular tissue routinely contains more SER, whereas the portal zone parenchyma contains more RER.

periportal cells in the 6-mo-old rats contained 61% more RER than similar hepatocytes in the 1-mo-old animals, but the surface area of this membrane subsequently declined until there was no measurable difference between the youngest and oldest rats.

Although none of the methods used to evaluate the Golgi membranes demonstrated significant differences in this parameter as a function of animal age or the sublobular location of the hepatocytes, the trend suggested that the periportal parenchyma contained larger volumes of Golgi-rich area and surface densities of Golgi membranes than the corresponding centrolobular tissue (Table II). The specific surface area data demonstrated that the individual centrolobular hepatocytes contained more Golgi-rich area and Golgi membranes than the portal zone parenchymal cells.

DISCUSSION

~80–85% of the intralobular liver tissue of rats consists of hepatocytes (6, 43, 44, 52). These cells exhibit differences in fine structure depending on their location within the liver lobule and such differences are difficult to detect by routine electron microscopy. Most stereological analyses of rat liver have not considered the influences of either sublobular location or animal age on parenchymal cell morphology. In the present study, the only sublobular difference in hepatocyte ultrastructure observed during qualitative examination was a greater number of cytoplasmic lipid droplets in the periportal zones. The only obvious age-related change was an apparent increase in the number and size of the dense bodies and a concomitant accumulation of lipofuscinlike and lipoidal material in these organelles. The incidence of

dense bodies containing crystalloid inclusions did not appear to be related to either the sublobular location of the liver cells or to animal age. We are unaware of any reports describing such structures or demonstrating their specificity to the Fischer 344 rat.

Influence of Sublobular Location on Hepatocyte Fine Structure

Loud (27) found no difference in hepatocyte volume between the centrolobular and portal zones of the liver lobule. Data from the present study demonstrated that the centrolobular hepatocytes are significantly larger than those in the periportal zone and, thus, support the findings of Jones et al. (20) which suggest that a gradient in parenchymal cell size exists across the liver lobule. This conclusion is supported by the data demonstrating similar lobular differences in the volumes of the cytoplasmic and ground substance compartments of the hepatocytes and the nuclear numerical densities.

The significantly greater volume of bile canalicular space in the portal parenchyma in comparison to the centrolobular zones agrees well with the observations of Jones et al. (19, 20) in a different strain of rats. Because bile flows from the centrolobular zone toward the portal triads, the periportal canaliculi are responsible for transporting larger volumes of bile than those more centrally located within the liver lobule. Furthermore, recent stereological studies on the rat liver during enhanced bile secretion (19) and total biliary obstruction (20) suggest that the portal hepatocytes are more active in bile secretion, whereas the centrolobular cells probably act in a reserve capacity and are recruited during hypersecretion.

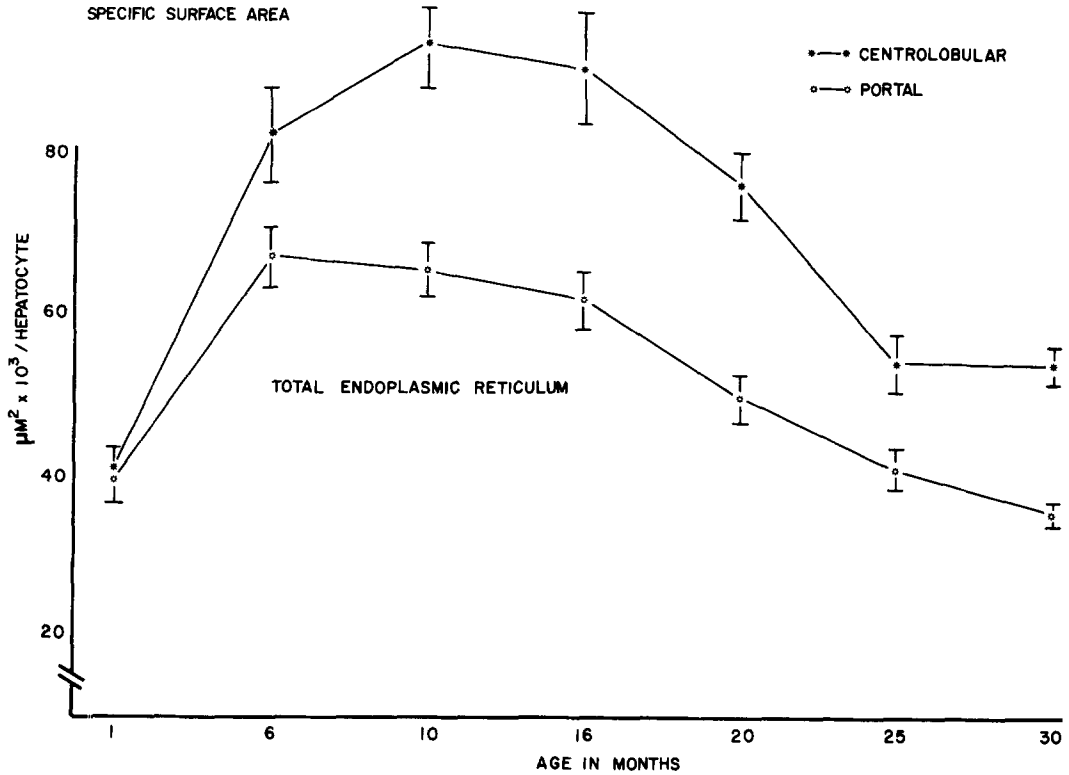

FIGURE 6 The surface area of total ER per average hepatocyte is plotted as a function of animal age and sublobular location. In all age groups, except 1 mo, the centrolobular cells contain significantly more ER than the portal hepatocytes. The amount of ER increases ~125% in the central zone cells between 1 and 10 mo of age, whereas the membrane surface area in the portal hepatocytes ceases to increase beyond 6 mo (60%). In both zones, the amount of ER declines during maturation and senescence until the values in the oldest rats approach those found in the 1-mo-old animals, particularly in the portal hepatocytes.

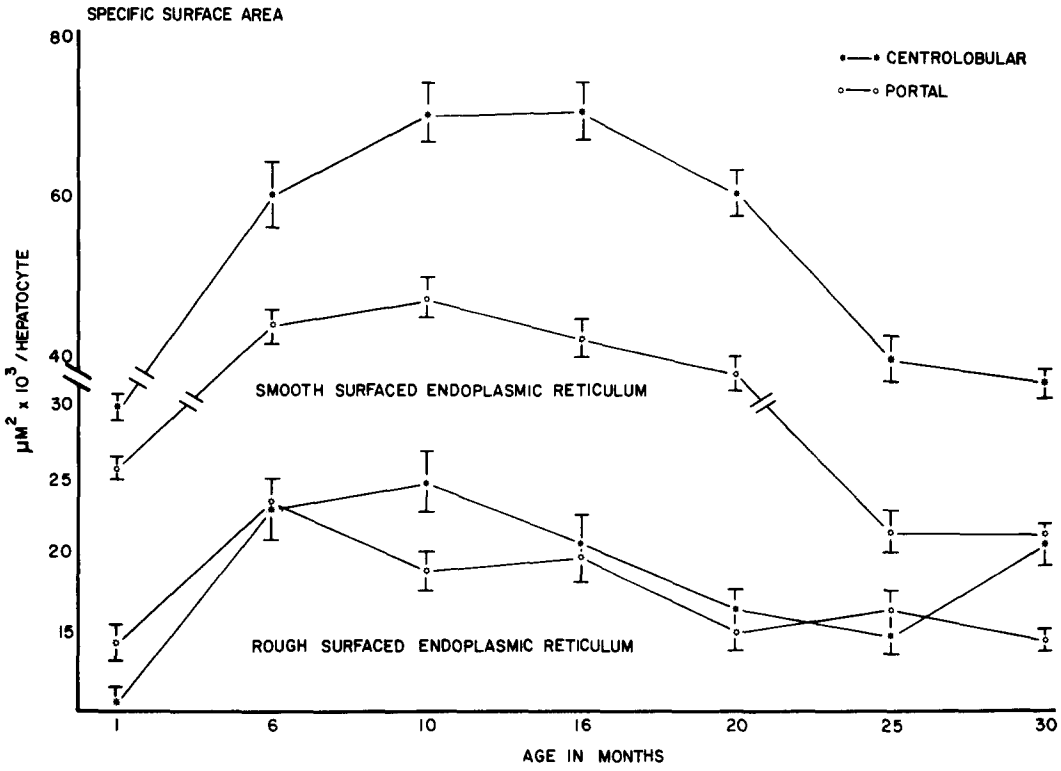

FIGURE 7 The specific surface areas of the SER and RER are plotted against animal age and sublobular location. The marked age-related changes in the surface area of the ER (see Fig. 6) are reflected in the amounts of SER in the various age groups. The centrolobular hepatocytes contain significantly more SER than the respective portal zone cells. Note that the changes in RER are minimal in comparison to the SER across the entire life-span and that there is no net loss of RER. Furthermore, the lobular gradient in RER distribution is much less apparent when the data are expressed as specific surface areas.

If the periportal cells are more active than those in the central zones during normal bile secretion, the larger volume and surface densities of Golgi complex in the portal parenchyma may be further evidence that this organelle plays a role in this hepatic function, a theory which has recently been proposed (12, 19, 20). Although the larger amounts of Golgi complex and Golgi-rich areas in the individual centrolobular hepatocytes appear to contradict this hypothesis, these specific data probably reflect the larger volume of these cells.

The larger volume of cytoplasmic lipid droplets in individual portal zone cells may result from the fact that these hepatocytes are exposed to higher concentrations of free fatty acids in the afferent portal blood in comparison to the centrolobular parenchymal cells. A similar lobular gradient in lipid deposition has been reported previously (20, 27). This lobular difference is even more marked in view of the fact that the portal cells are significantly smaller than the centrolobular hepatocytes.

The lobular gradient in mitochondrial volume density confirms the findings of Loud (27) and Jones et al. (20). However, Loud (27) reported that the individual periportal hepatocytes contained significantly greater volumes of this organelle than corresponding centrolobular cells. Our data, and those of Jones et al. (20), for similarly aged rats demonstrate no significant sublobular differences in the specific volume of hepatocellular mitochondria. At present, we are unable to account for this discrepancy, although differences in the fixation procedures between our studies and that of Loud cannot be eliminated.

The minor variations in the volume densities and specific volumes of the microbodies and dense bodies were not sufficient to demonstrate a lobular gradient in the distribution of these organelles. The differences between our data and those of others for these stereological parameters are

within the range of statistical error (20, 27).

The finding of a greater surface area of ER in the centrolobular hepatocytes in the present study is consistent with the results of Jones et al. (20) and Loud (27). This lobular gradient in the distribution of the ER is attributable to a similar gradient in the SER. The fact that certain drugs, such as sodium phenobarbital, and hepatotoxins, such as carbon tetrachloride, which are metabolized by enzymes associated with the SER, first affect the centrolobular zones (7, 36), suggests a functional correlate to this sublobular distribution of membrane. The sublobular distribution of the RER was much less marked, although the portal parenchyma consistently contained more of this membrane than the corresponding centrolobular tissue. At the present time there is no definitive evidence for a correlative lobular gradient in hepatic protein synthesis (26). These quantitative data conflict with those of Loud (27) who found more RER in the centrolobular zones of livers of young rats. The reason(s) for this contradiction has not been resolved, but variations due to differences in animal strain and sex cannot be excluded.

Influence of Animal Age on Hepatocyte Fine Structure

Previous studies of age-related changes in hepatic ultrastructure described a number of alterations (see reference 43 for a brief review). Most of these studies were inconclusive, compared only two age points, and did not include quantitative morphological data. To appreciate the effect of aging on structural and/or functional parameters, a number of age points should be evaluated within a single population. Because there is no explicit definition of a senescent animal, such studies should be designed to examine the entire lifespan, including the periods of development, maturation, and senescence.

Histological and ultrastructural evaluations of the rat liver showed: (*a*) an increase (3, 4, 49), (*b*) a decrease (37), and (*c*) no change (47) in hepatocyte size as a function of animal age. Ross (37) conducted the only extensive analysis of the effect of aging on hepatocyte size and demonstrated that the volume of rat liver parenchymal cells increased during the first 12 mo of life, followed by a decrease during the subsequent 12 mo. However, Pieri et al. (32) reported that rat hepatocytes continued to increase in volume through 27 mo of age. The data from the present study agree well with those of Ross (37) and, furthermore, demonstrated similar age-related changes in the specific volumes of the hepatocytic cytoplasmic and ground substance compartments, as well as complementary changes in the nuclear numerical density. In spite of the reported increase in hepatocyte nuclear ploidy with age in the rat (55), the relative number of binucleate parenchymal cells, the nuclear numerical density, and the nucleocytoplasmic volume ratio were similar in the youngest and oldest animals.

With the exception of the dense bodies, hepatocyte fine structure was remarkably similar in the youngest (1 mo) and oldest (30 mo) rats. Real age-dependent losses in the volumes and/or surface areas of hepatocellular organelles occurred only between maturity (10–16 mo) and senescence (25–30 mo). Most of the changes in the specific parameters, i.e., volumes or surface areas per individual hepatocyte, merely reflected the age-related alterations in cell size. However, changes in both the relative and specific values were considered indicative of real age-dependent alterations.

A number of studies reported a decrease in the number of mitochondria (17, 31, 45, 46, 48), an increase (52) or no change (17) in average mitochondrion volume, an increase in the number of microbodies (48), and the disruption and loss of RER (31, 32) in the livers of aged animals. Pieri et al. (32) reported finding a greater number and volume of mitochondria in the livers of 27-mo-old rats in comparison to 1-mo-old animals, as well as swollen mitochondria in the oldest rats. The reduction in mitochondrial volume density observed in the present study suggests that this organelle undergoes a net loss with age and supports the semiquantitative evidence demonstrating an age-dependent decline in the activities of certain hepatic respiratory enzymes (56). Therefore, mitochondrial dysfunction during senescence appears to be due to a loss of volume rather than to a qualitative alteration in organelle structure.

The fact that Pieri et al. (32) evaluated dense bodies and microbodies collectively precludes any definitive conclusions regarding quantitative age-related changes in these two organelles. Tauchi et al. (48) reported an increase in the number of hepatic microbodies in old rats. Although we did not determine the numerical density of this organelle, our data demonstrate a net loss in the volume of microbodies between mature and senescent animals. This finding is certainly not in disagree-

ment with that of Haining and Legan (16), who reported that hepatic catalase activity was generally lower in older rats.

The net increases in both the volume density and specific volume of dense bodies in rats between 1 and 30 mo of age support previous qualitative electron microscopic evidence demonstrating that the number of hepatocyte lysosomes increases with age (23, 37). The age-dependent increase in the amounts of electron-opaque and lipoidal material in the dense bodies probably reflects the accumulation of lipofuscin and the formation of residual bodies.

The observation that drug-induced proliferation of SER membranes accompanied an increase in hepatic microsomal drug-hydroxylating activity (35) provided the basis for correlating studies on induced enzyme synthesis and membrane biogenesis. Kato and Takanaka (21) and others (11, 39) found that the activity of liver microsomal NADPH cytochrome *c* reductase and the amount of cytochrome P-450 increased more markedly in young rats than in older animals after phenobarbital stimulation. Evidence from studies such as these suggested that the livers of senescent animals had a reduced capacity to metabolize drugs and other moieties, such as steroids and hepatotoxins.

Previous stereological studies from this laboratory showed that the surface area of the hepatic SER increased linearly in rats between 3 and 16 mo of age (43). The present data confirmed these earlier results and also demonstrated a significant age-dependent loss of SER between maturity and senescence. However, Pieri et al. (32), in a study involving three age groups, reported that the amount of hepatic SER declined significantly between 1 and 12 mo of age, but subsequently increased until 27-mo-old rats contained significantly greater levels of this hepatocellular membrane than the youngest animals. Because we employed a smaller reference volume than these investigators, i.e., hepatocyte ground substance vs. total hepatic parenchyma, our estimates of membrane surface areas should be somewhat greater. Still, differences in the mode of expression of data cannot account for such variations in results, especially when similar stereological methods were employed.

Although we have noted minor differences in several hepatocellular stereological parameters among similarly aged Holtzman Sprague-Dawley, Charles River Sprague-Dawley, and Fischer 344 strain male rats, it seems unlikely that variations in animal strain can account for such a discrepancy. Pieri et al. (32) also included the Golgi membranes in their estimates of the SER, but the contribution of these membrane profiles to the total SER surface area is insignificant. Therefore, we interpret our results to represent a real, rather than apparent, age-dependent loss of SER and suggest that this change is a morphological correlate of the reported reduced hepatic capacity to metabolize drugs with age.

Pieri et al. (32) reported a steady decline in the amount of hepatic RER in rats between 1 and 27 mo of age (~75%). However, our results showed no significant loss in RER surface density, and the specific surface area estimates reflected the changes in hepatocyte volume, suggesting that there is no net loss of hepatic RER with age. Because we employed a much larger sample size than Pieri et al. and because our animals were obtained from a carefully controlled colony maintained solely for aging studies, we suggest that our data correctly reflect the age-dependent alterations in the amounts of RER and SER as well as in hepatic fine structure in general.

The present stereological evidence demonstrates that a continuous gradient in hepatocellular fine structure exists across the entire liver lobule. Portal tissue contains fewer hepatocytes and greater volumes of biliary space, RER, cytoplasmic lipid, Golgi complex, and dense bodies than the centrolobular parenchyma. Hepatocytes closer to the portal triad are smaller, usually contain less total ER and SER and more lipid than cells nearer the central vein. Lobular differences in the distributions of SER and Golgi complex may represent morphological correlates of functional gradients in drug metabolism or bile secretion.

Age-related alterations in hepatic fine structure occur regardless of the sublobular location of the hepatocytes. The liver tissue of older animals generally contains a similar or larger number of hepatocytes, similar or smaller amounts of mitochondria, total ER, SER, microbodies, and Golgi complex, and similar or larger volumes of dense bodies and biliary space than tissue from very young or mature rats. The volumes of most hepatocyte organelles increase during development and subsequently decrease between maturation and senescence, thus reflecting the changes in individual cell volume. In fact, the fine structure of hepatocytes in senescent rats is very similar to that found in young animals. With the exception of the specific volume of dense bodies, which

increases in both lobular zones, the age-related decline in organelle volumes or surface areas results in cells of senescent rats containing similar or smaller amounts of mitochondria, total ER, SER, microbodies, lipid, and Golgi complex in comparison to those in the youngest age group.

Although periportal hepatocytes are generally considered to be "younger" than centrolobular cells, no selective age-related effects on either lobular zone were apparent in the present study. Evidence for the theory that portal cells are "younger" is largely derived from studies on regenerating livers, which do not represent a normal hepatic condition. Furthermore, Pieri et al. (33) demonstrated that the fine structure of newly regenerated hepatocytes of senescent rats is quantitatively similar to that of resting cells in animals of the same age. Thus, the age of the animal, and not the age of the cell per se, appears to be the important factor in determining age-dependent changes in hepatic fine structure.

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