HEPATOCYTE INNERVATION IN PRIMATES

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ABSTRACT

The efferent innervation and some characteristics of nerve fibers of the liver lobule in the tree shrew, a primate, are described. Nerve endings on hepatocytes were encountered regularly and were determined to be efferent adrenergic nerves. Transmission electron microscopy revealed nerve endings and varicosities in close apposition to the hepatocytes adjacent to the connective tissue of the triads as well as within the liver lobule in the space of Disse. Fluorescence microscopy indicated the existence of adrenergic nerves with a similar distribution. Autoradiography of the avid uptake of exogenous [^{3}H]norepinephrine indicated that all intralobular nerves are potentially norepinephrinergic (adrenergic). Chemical sympathectomy with 6-OH-dopamine resulted in the degeneration of all intralobular liver nerve fibers as revealed by fluorescence microscopy and electron microscopy. Substantial regeneration occurred after 60–90 days but was not completed by that time. Some nerves were also observed in close association with von Kupffer cells and endothelial lining cells. The functional significance of the efferent liver innervation is discussed.

The presence of nerves in the mammalian liver is generally acknowledged and has been studied by various morphological and physiological methods, but the precise distribution of nerves to the liver lobules or to the hepatocytes has not been determined. Riegele (33) described intralobular innervation in the liver of cat, rabbit, and human and confirmed the results of earlier studies of the last century (20, 22, 24, 25, 30, 31). Some recent light microscope studies on liver innervation with the use of various preparation and staining methods have been directed towards the confirmation of hepatocellular innervation (3, 23, 35, 37). However, there are other reports in which no evidence for such innervation has been shown (1, 6, 28, 41).

Although the liver has been the subject of numerous ultrastructural studies, the only electron microscope study that we have found is the publication of Yamada (45) (see also reference 44) which describes nerves ending on hepatocytes adjacent to connective tissue of the portal triads in the mouse liver. Recently developed fluorescence microscope techniques for nerves have been used as a more adequate method for demonstrating liver innervation. Nevertheless, the opinion is still predominant that nerves are found only in the connective tissue of the triads and along blood vessels but not within the lobules (see reference 41).

Recent physiological studies suggest that afferent nerves from the liver may be involved in osmoreception (2, 17, 26), but this interpretation has been questioned (4, 14). There is also some evidence (29) indicating the presence of efferent liver nerves that are involved in chemoreception.

Stimulation of efferent nerve fibers (15, 16) was reported to affect blood flow through the liver of

various species. Furthermore, some physiological studies (9, 27) have suggested innervation of hepatocytes by the splanchnic nerve. Some important evidence suggesting efferent liver innervation was shown by the biochemical studies on the influence of liver nerves on liver cell metabolism (8, 9, 34). In the toad, it was reported that stimulation of the splanchnic nerves in the isolated liver preparation caused a release of glycogen into the vascular system (27).

The present report is an ultrastructural and fluorescence microscopical study of nerve fiber distribution in the liver of the primate, *Tupaia belangeri*. In this species, the liver lobule is supplied with adrenergic nerves which form close contacts with some hepatocytes in the peripheral zone of the lobule where parenchymal cells as well as sinusoidal lining cells and von Kupffer cells appear to be innervated. A preliminary report of some of these observations has been published (12).

MATERIALS AND METHODS

Liver samples from 23 adult tree shrews, *T. belangeri*, of both sexes, weighing 120–180 g, were used. For electron microscopy, six control animals were fixed by vascular perfusion similar to a method described elsewhere (13). In brief, the procedure involves the cannulation of the abdominal aorta and initial perfusion of the vascular system for 60 s with a solution containing 0.1% procainhydrochloride, 0.01% heparin, 2.5% polyvinylpyrrolidone (PVP) mol wt 40,000 (PVP, 40,000; Sigma Chemical Co., St. Louis, Mo.) in 0.9% NaCl and adjusted to pH 7.2 with HCl. This is immediately followed by perfusion of the fixative containing 1.5% formaldehyde, 1.5% glutaraldehyde, 2.5% PVP 40,000 in 0.09 M Na-phosphate buffer at pH 7.2. With the system employed, about 500 ml of the fixative were perfused in about 5–8 min. After this period, the viscera was removed and placed in the same fixative for storage. Soon after initial fixation, liver blocks of about 1 × 1 × 5 mm were rinsed twice in phosphate buffer and osmicated in 1% osmium tetroxide in phosphate or cadodylate buffer, or in a mixture of 1.5% potassium ferrocyanide and 1% osmium tetroxide in distilled water (19). The blocks were then rinsed several times in maleate buffer at pH 5.2 and stained *en bloc* in 1% uranyl acetate in the same buffer for about 1 h. All fixation steps were done at room temperature. After final rinses in maleate buffer, the tissues were dehydrated in cold ethanol solutions and embedded in a mixture of Epon and Araldite (21). Thin sections were stained with uranyl acetate and lead citrate (42) and examined in the electron microscope.

Experimental animals for chemical sympathectomy were treated according to the method of Thoenen and Tranzer (39) and Tranzer and Thoenen (40). The animals were injected intraperitoneally with six doses each of 4 mg of 6-OH-dopamine (Sigma) in one group and 10 mg in another group at 12-h intervals. After the last injection, two animals were sacrificed after 1 day, three animals after 3 days, and one each after 42 days, 67 days and 82 days. All animals were fixed by perfusion and examined as described.

For autoradiography, 3.0 mCi L-[^{3}H]norepinephrine, (7-^{3}H (N)) (sp act 15 Ci/mmol, New England Nuclear Corp., Boston, Mass., NET-277) was injected 1 h before perfusion fixation, and the tissues were prepared as for normal thin-section electron microscopy. Thin sections were coated with Ilford L4 emulsion by the loop method (5) and exposed for 21 days. For development of exposed silver grains, Kodak Microdol X was used. Autoradiographs were stained with lead citrate. Autoradiography was also carried out in three animals after injection of 2.5 mCi [^{3}H]epinephrine, 2.5 mCi [^{3}H]dopamine and 2.5 mCi[^{3}H]serotonin. No significant labeling was observed in these experiments.

Five animals were processed for fluorescence microscopy according to the methods of Falck et al. (10). These livers were excised after decapitatin of the tree shrew, and small 4- to 5-mm samples were frozen at −120°C Freon liquified with nitrogen. The frozen blocks were vacuum dried at −60°C and then exposed to formaldehyde vapor at 70% humidity for 12 h. After embedding in paraffin, 5- to 10-μm sections were cut and examined in a Leitz Orthoplan fluorescence microscope with a UV light source and Leitz BGB 20 filter. For fluorescence microscopy, two control animals and three animals after treatment with 6-OH-dopamine were used. The treatment was six doses of 10 mg of 6-OH-dopamine intraperitoneally at 12-h intervals. The animals were sacrificed 24 h, 4 wk, and 12 wk after the last injection.

To extend the present observaions, liver samples of human and other primates were examined by electron microscopy and fluorescence microscopy. Micrographs of nerves from a monkey, *Macaca mulatta*, are included in this report.

RESULTS

Fluorescence Microscopy

Fluorescence microscopy of the frozen-dried sections reveals numerous nerve plexuses, in the *Tupaia* hepatic triads, as a dense network of intensely green-fluorescing structures particularly around the branches of the hepatic arteries (Fig. 1). From these nerve plexuses, numerous branches enter the hepatic lobule and extend about halfway towards the central vein (Figs. 1 and 2). These nerves are in the space of Disse and thus outline the hepatic sinusoids (Fig. 2) and form varicosities as well as bulbous endings. This method reveals the extensive distribution of nerves and their close approximation to many parenchymal cells and sinusoidal lining cells, but

their precise or intimate relationship to liver cells cannot be resolved by this method.

Fluorescence Microscopy after Chemical Denervation

The fluorescence due to nerves disappears in 1–3 days after 6-OH-dopamine treatment (Fig. 3). Animals similarly treated but allowed to regenerate for 67 and 82 days reveal nerves growing from the triad region into the liver lobules. Although nerves are found within the lobules after this period, they are localized around the triad region and not deep within the lobule (Fig. 4).

Electron Microscopy

Electron microscopy confirms the presence of nerves in the connective tissue of the triads and many small branches within the hepatic lobule in the space of Disse (Fig. 5). The intralobular nerves are unmyelinated and usually have no ac-

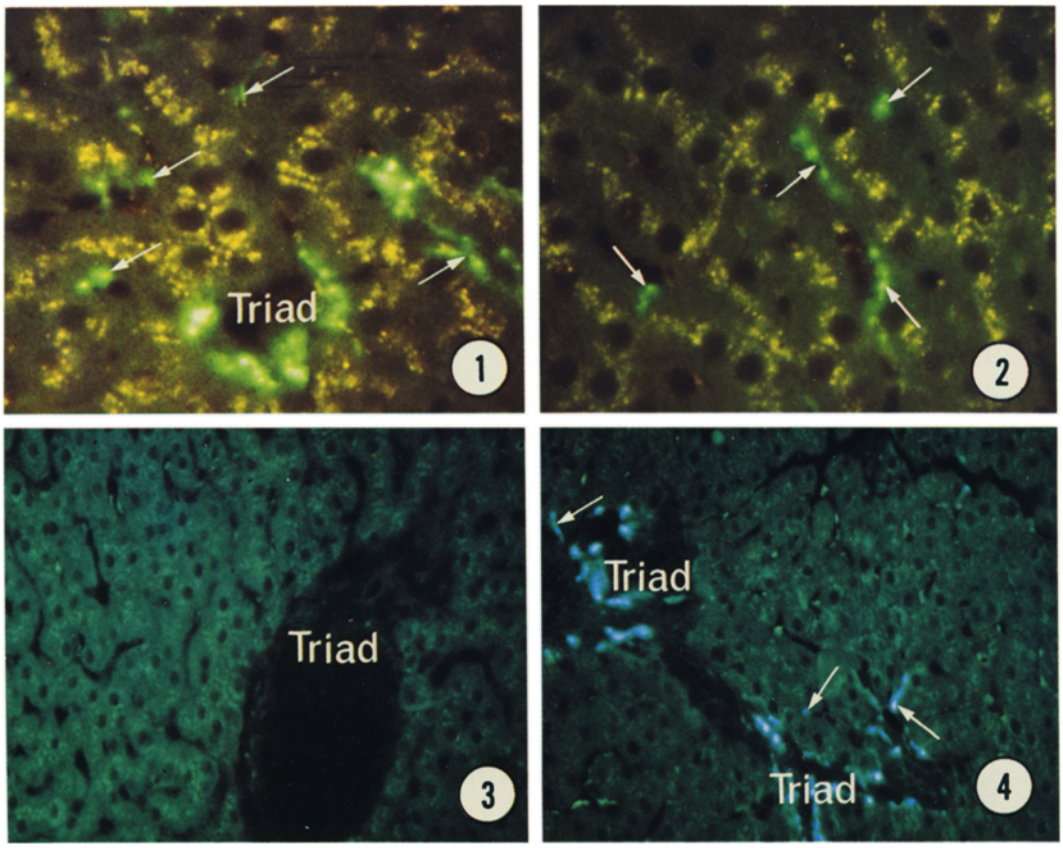
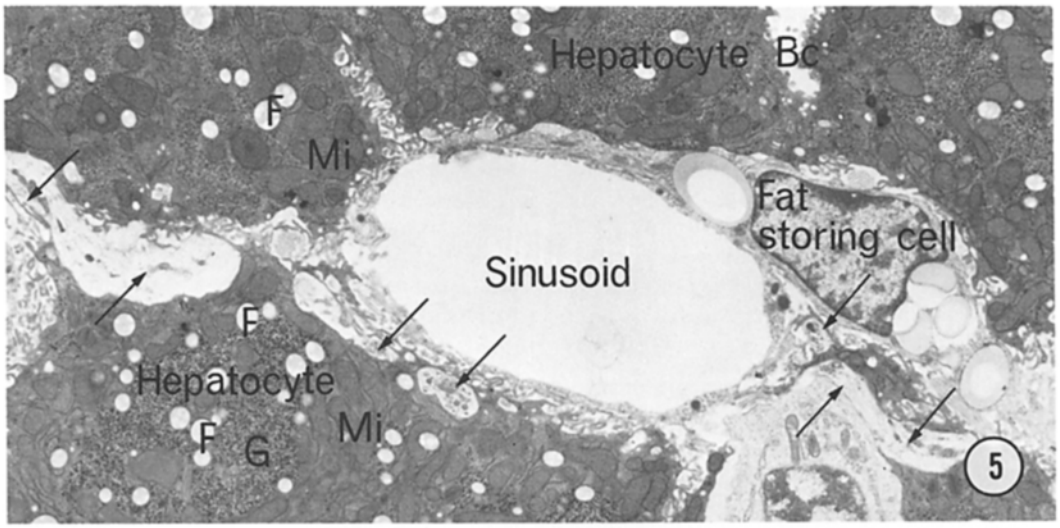


FIGURE 1 Fluorescence photomicrograph of frozen-dried liver sections of control *T. belangeri* showing green-fluorescing nerve fibers in the lobule adjacent to the portal triad and within the lobule along sinusoids (arrows). × 400.

FIGURE 2 Control liver of *T. belangeri* showing by fluorescence microscopy adrenergic nerve fibers deep in the liver lobule. Several varicosities are seen (arrows). × 400.

FIGURE 3 Fluorescence photomicrograph of *Tupaia* liver after chemical denervation (six doses of 10 mg of 6-OH-dopamine at 12-h intervals; fixation 3 days after the last injection). Fluorescent nerve endings or processes are no longer found within the liver lobules or triad region. × 200.

FIGURE 4 *Tupaia* liver after chemical denervation as in Fig. 3, except that the animal was fixed 82 days after the last injection. Numerous fluorescing nerve processes in the periphery of the liver lobule (arrows). × 200.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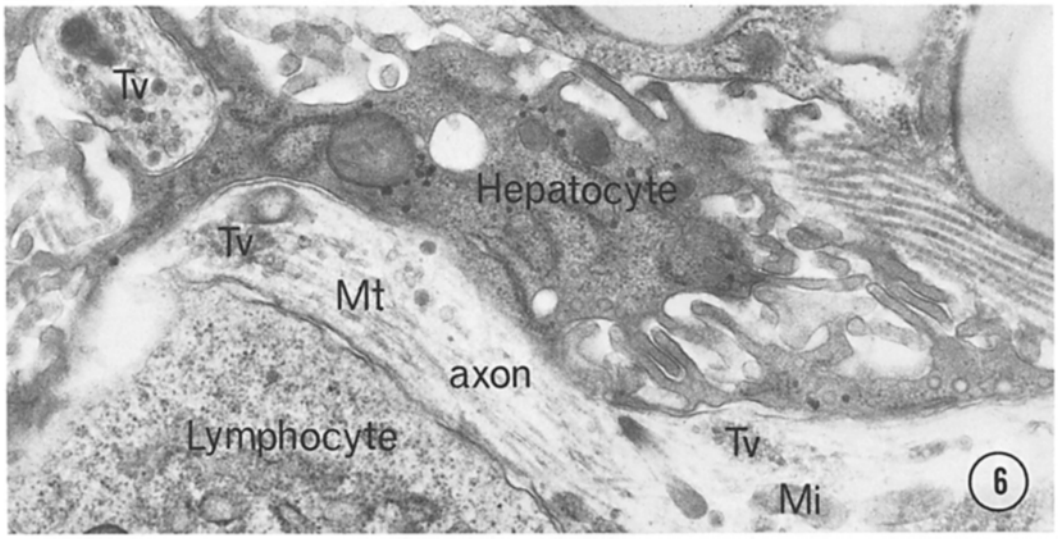
5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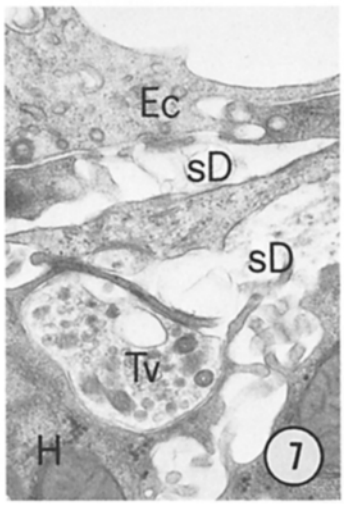
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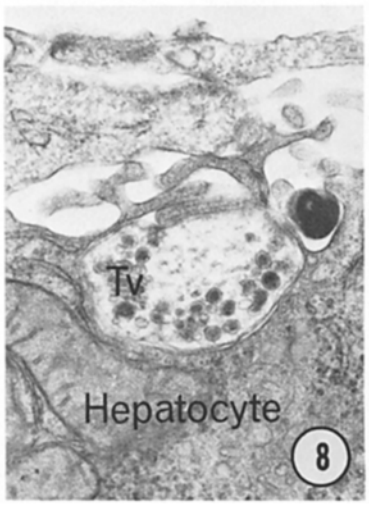
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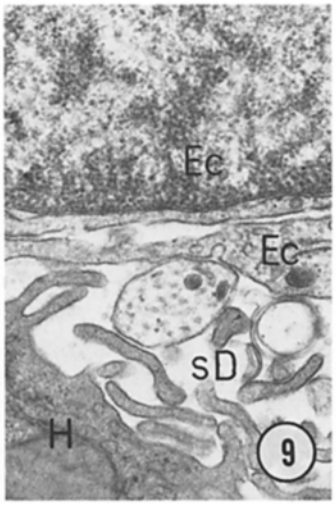
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companying Schwann cell investments (Figs. 6–9). These bare nerve processes have varicosities where the neurolemma is in close contact (separated by a space of about 20 nm) with liver cells (Figs. 7 and 8) and, less commonly, with sinusoidal lining cells (Fig. 9). Some of these swellings are at the terminals of the nerve processes. The varicosities and endings contain clusters of transmitter vesicles of three types: (*a*) large, dense-cored vesicles 80 nm in diameter with a close-fitting membrane, (*b*) smaller, dense-cored vesicles 40 nm in diameter with a punctiform core, and (*c*) clear vesicles 40 nm in diameter (Figs. 7 and 8).

The nerve processes course in the space of Disse among the collagen fibers and cell processes. The varicosities and endings lie in grooves or notches of the liver cells or on their surface (Figs. 7 and 8). At these sites, the nerve is in close apposition to the liver or sinusoidal lining cell, and a space of about 20 nm separates the plasma membranes. However, no distinct membrane thickening or specialization in these areas has been observed.

Electron Microscopy after Chemical Sympathectomy

Marked changes in the nerve fibers are apparent in thin sections of *Tupaia* liver specimens fixed 24 h after the 3-day treatment with 6-OH-dopamine. The axons of nerves from the hilus of the liver lobule are swollen as are some mitochondria and cisternae of the endoplasmic reticulum (Figs. 10 and 11). Some nerve fibers also contain myelin figures, dense bodies, and clusters of small, dense inclusions. This type of change is common in all fibers found in the parenchymal tissue, but in the connective tissue a number of the nerve fibers in the triad area appear normal and apparently are unaffected by the 6-OH-dopamine treatment. Fibers in the liver lobule are no longer recognizable and are replaced by myelin bodies (Fig. 13).

In livers of animals fixed 3 days after the treatment with 6-OH-dopamine, intact nerve fibers in the liver lobule and triad region have virtually disappeared or are greatly disrupted and cannot be identified with certainty. To trace the retrograde degeneration of the liver nerves after the treatment for chemical sympathectomy, we examined the nerves at the hilum of the liver and along the main intrahepatic blood vessels. These observations indicate that degenerative changes occur in about 20% of these axons (Figs. 10, 12, and 14). In the affected fibers, structures such as microtubules, transmitter vesicles, and mitochondria are replaced by an accumulation of dense inclusions and myelin figures (Fig. 14). The remaining intact axons appear to be unaltered by the treatment.

In the animal sacrificed 67 days after the last injection of 6-OH-dopamine, nerve fibers are found within the parenchyma of the liver (Fig. 15). These nerves are predominantly localized in or near the connective tissue of the portal triads and are less frequently found deep within the lobules. In these specimens, the varicosities often contain a large number of transmitter vesicles but are otherwise similar to the control liver. The myelin figures and dense inclusions found in nerve fibers of animals fixed soon after 6-OH-dopamine treatment are not evident; and nerve-liver cell or nerve-Kupffer cell close contacts are similar to those found in untreated animals. In livers from the animal fixed 82 days after 6-OH-dopamine treatment, the nerve fibers are found deeper in the lobules (Figs. 15–18).

FIGURE 5 Electron micrograph of nerve fibers in the liver lobule of a control *Tupaia*, similar to the region shown in Fig. 2. The nerve process has several varicosities (arrows). Surrounding hepatocytes contain many mitochondria (*Mi*), fat droplets (*F*), glycogen (*G*), and a bile canaliculus (*Bc*). × 3,000.

FIGURE 6 Higher magnification of an axon from the lower right of Fig. 5. Accumulations of transmitter vesicles (*Tv*), microtubules (*Mt*), and mitochondria (*Mi*) are seen within the axon, which is bordered by a hepatocyte and a lymphocyte. × 21,500.

FIGURE 7 A varicosity along a liver nerve fiber in transverse section. Cytoplasmic extensions of a hepatocyte (*H*) almost completely surround the varicosity. Endothelial cell (*Ec*), space of Disse (*sD*), transmitter vesicles (*Tv*). × 16,000.

FIGURE 8 Varicosity of a liver nerve similar to that in Fig. 7. Dense-cored vesicles (*Tv*) predominate in this example. × 21,500.

FIGURE 9 Liver nerve fiber in the space of Disse (*sD*) showing microtubules. The nerve is more intimately associated with the endothelial cell (*Ec*) than with the hepatocyte (*H*). × 21,500.

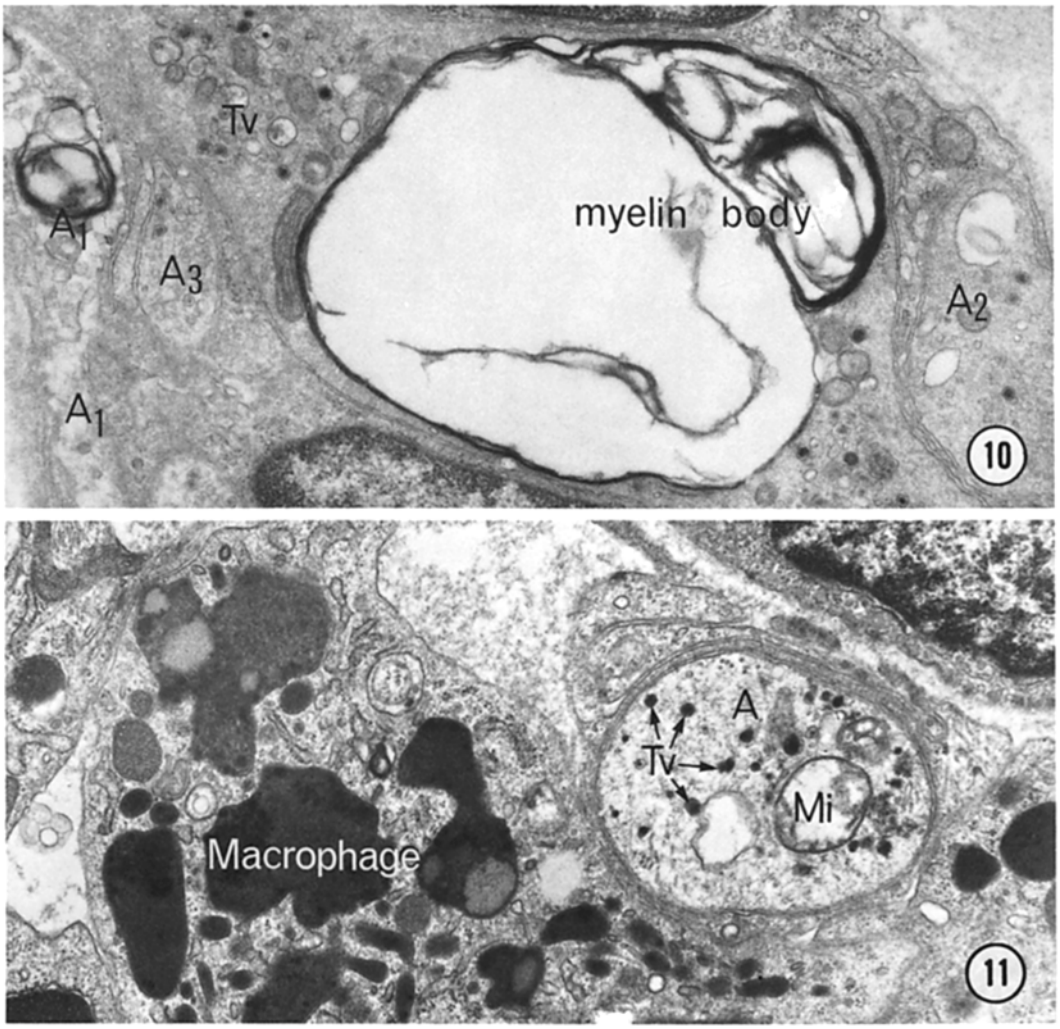

FIGURE 10 Liver nerve fibers after chemical denervation with 6-OH-dopamine and fixed 3 days after the last injection. This micrograph is from the region adjacent to the arteria hepatica propria. A swollen myelin body in a large adrenergic nerve varicosity with transmitter vesicles (*Tv*) is shown. Axons affected to varying degrees (A_1, A_2, and A_3) surround the varicosity. × 19,000.

FIGURE 11 Appearance of axons in the connective tissue of the portal triad after chemical denervation with 6-OH-dopamine and fixation 1 day after the last injection. The axon (*A*) is slightly swollen and contains dense transmitter vesicles (*Tv*) and a swollen mitochondrion (*Mi*). A macrophage surrounds the degenerating axon. × 11,500.

Autoradiography

When *Tupaia* were injected with radioactive norepinephrine, autoradiography of liver tissue clearly reveals the uptake of this label in virtually all nerve varicosities and endings observed within the liver lobule. Fig. 21 shows a region of three labeled nerves infolded into grooves in hepatic cells. A relationship of the silver grains to the transmitter vesicles containing varicosities is apparent. The uptake of labeled tracer substance is especially striking in longitudinal sections through nerves (Fig. 19). Radioactivity in small nerves abutting sinusoid lining cells is also evident (Fig. 20). In fact, the localization of radioactivity is so selective that the silver grains in the autoradiographs serves to locate nerves that are overlooked in unlabeled tissue. Attempts to label these

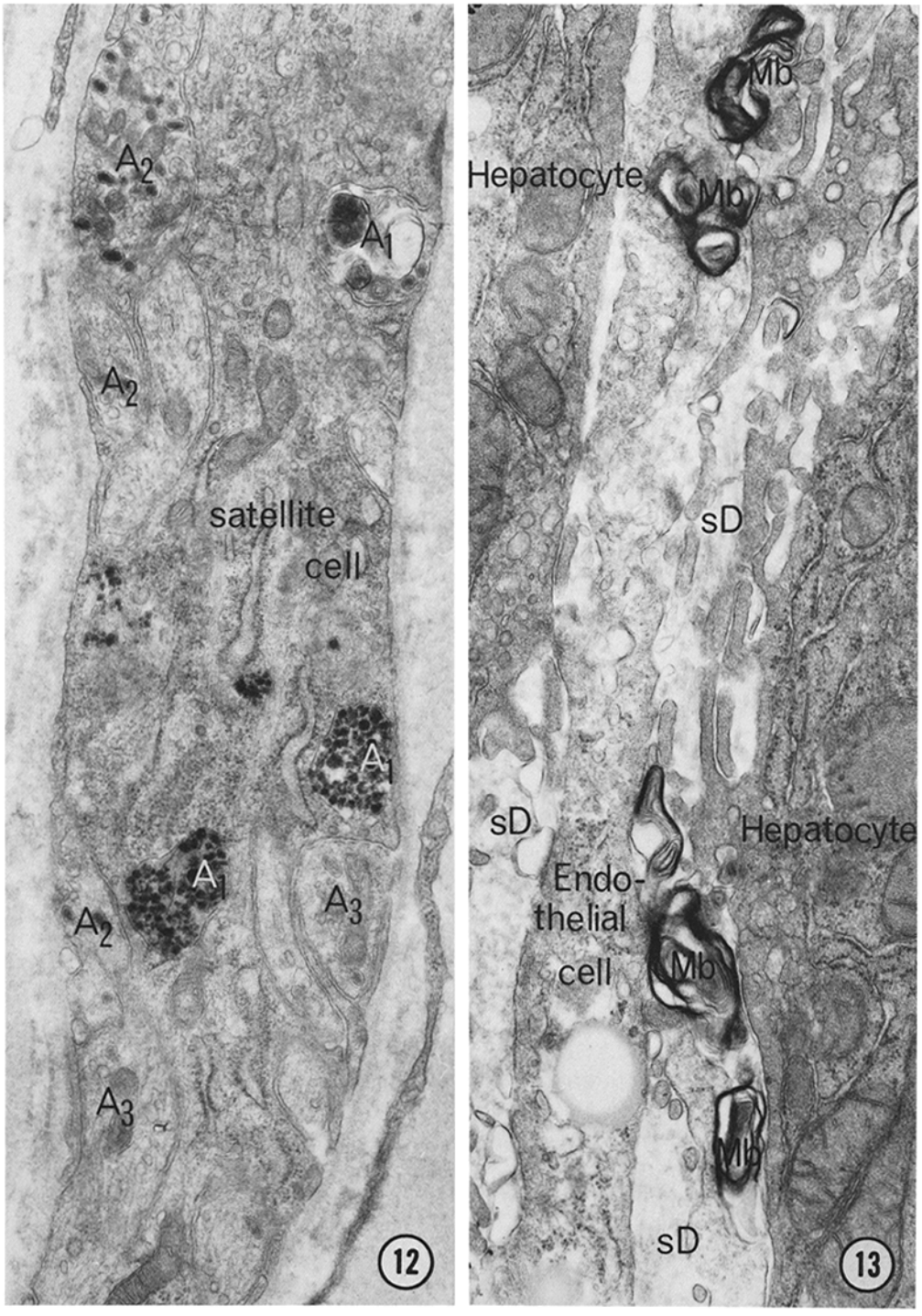

FIGURE 12 Liver nerve along a large intrahepatic blood vessel with typical signs of degeneration of sympathetic axons (A_1) after 6-OH-dopamine treatment and fixation 3 days after the last injection. Unaffected normal axons with dense-cored vesicles (A_2) and clear vesicles (A_3) are still present. × 22,000.

FIGURE 13 Sections of intrahepatic nerve fibers in the space of Disse (*sD*) after chemical denervation (same animal as in Fig. 11). The nerve has apparently broken down into myelin bodies (*Mb*). × 23,500.

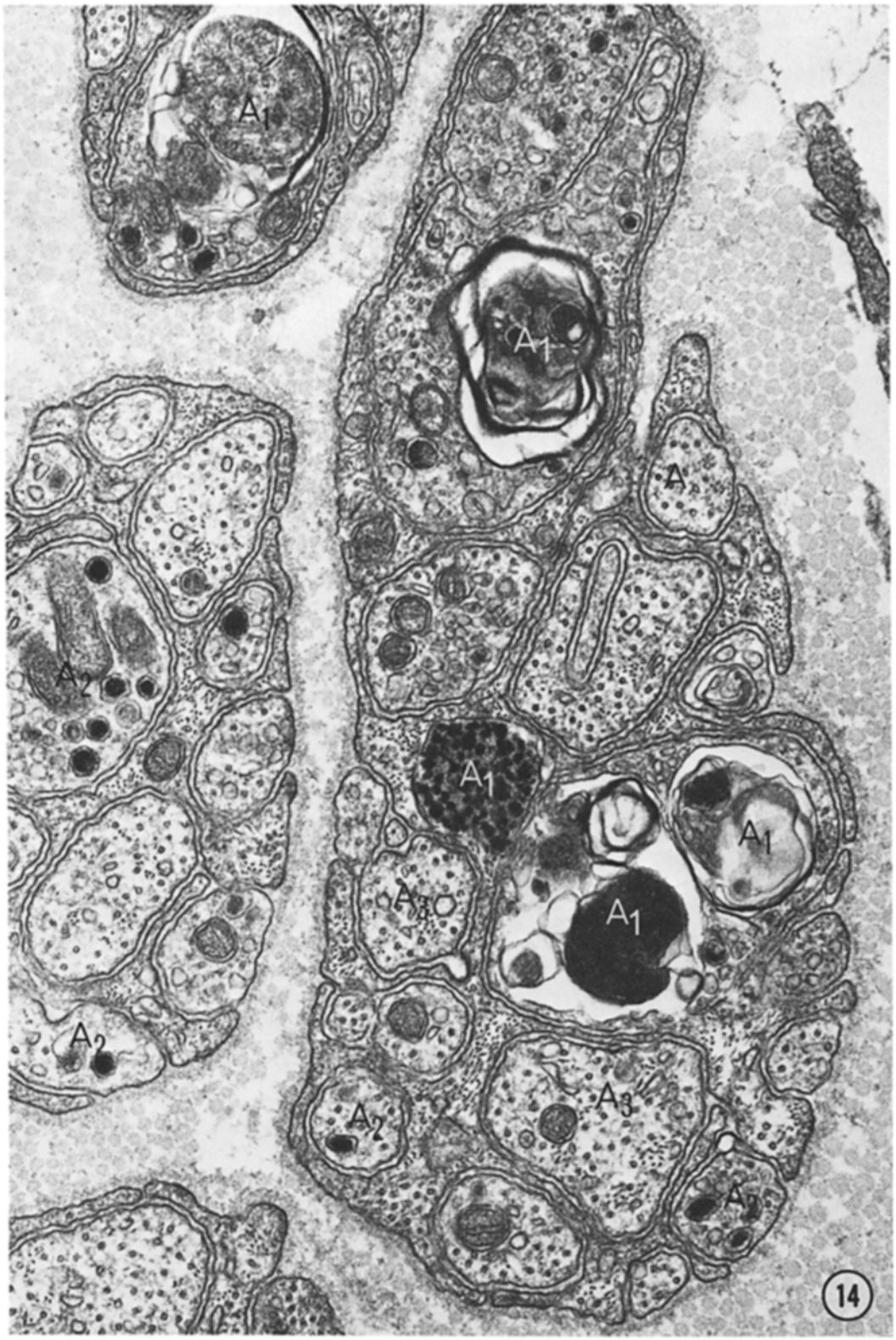

FIGURE 14 Part of the hepatic nerve along the arteria hepatica communis and the ductus choledochus after chemical denervation (same animal as in Fig. 11). Some nerve fibers (A_1) have degenerated while other axons (A_2) with dense-cored vesicles as well as the axons without transmitter vesicles (A_3) seem to be unaffected. Microtubules and microfilaments are present in these nerve fibers. × 60,000.

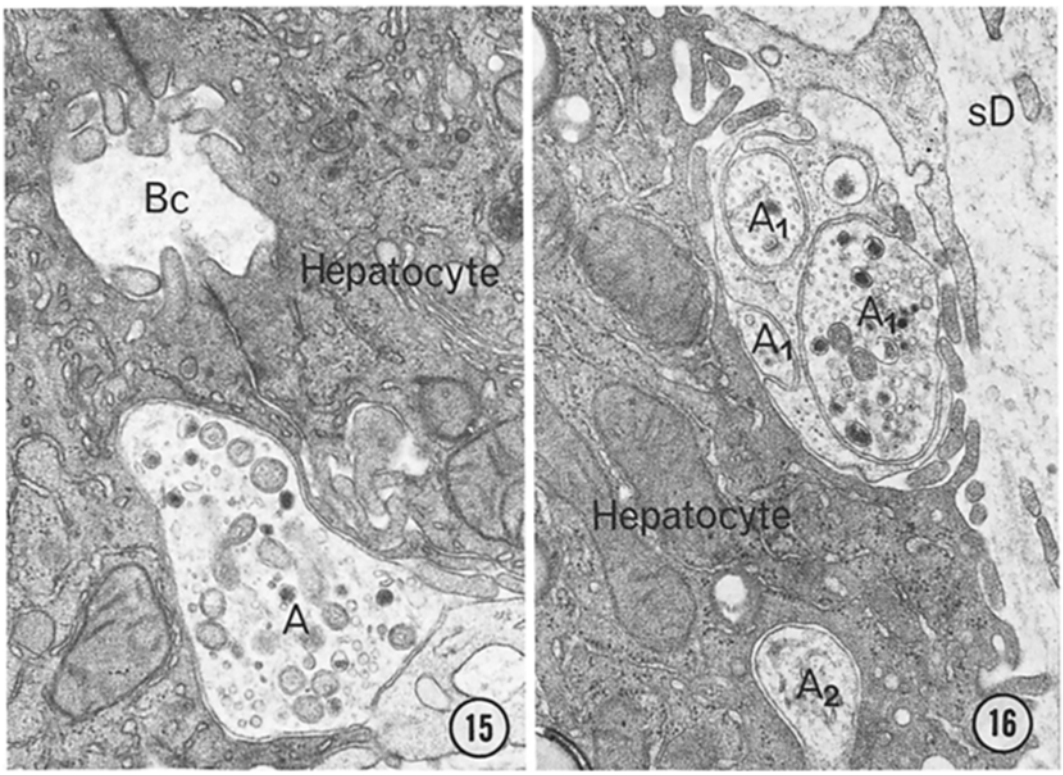

FIGURE 15 Intralobular nerve varicosity (*A*) with dense-cored vesicles and small mitochondria 82 days after chemical denervation with 6-OH-dopamine. It is surrounded by hepatocytes and is close to a bile canaliculus (*Bc*). × 34,000.

FIGURE 16 Sprouting nerves along the space of Disse (*sD*) after chemical denervatin (same animal as in Fig. 15). Some of the nerves (A_1) are supported by a satellite cell while another (A_2) is in contact with hepatocyte. × 34,000.

nerves with radioactive epinephrine, dopamine, or serotonin resulted in no apparent uptake of these biogenic amines.

Liver Parenchymal Innervation in the Monkey M. mulatta

In *Macaca,* liver nerves are less abundant than in *Tupaia*. Those liver cells at the periphery of the lobule adjacent to the triads frequently have associated nerve fibers (Fig. 22). The intralobular axons (Figs. 23 and 24) are similar to those seen in *Tupaia*. Sometimes, they contain large, dense-cored transmitter vesicles which are irregular or ovoid in shape and densely packed. The general aspect of liver innervation in *Macaca* is similar to that in *Tupaia*.

DISCUSSION

Though few organs have been studied so extensively as the liver, relatively little has been established or generally recognized regarding the innervation of the liver parenchymal cells. In fact, few investigators realize that some liver cells are indeed innervated. In this original study in 1875, von Kupffer (43) set out to investigate nerves in the liver lobule, but he was unable to demonstrate nerves and instead described the "Sternzellen" which is now known as the Kupffer cell. He writes, "Im Verlauf der andauernden und immer noch vergeblichen Bemühungen Nerven in den Leberläppchen nachzuweisen. . . ." In his review of liver microscopic morphology in 1932, Pfuhl (31) refers to the publication of Riegele (33) as the only convincing demonstration of innervation in the liver of cat, rabbit, and human. The latter study described nerve endings on hepatocyes and Kupffer cells within the liver lobule.

The first, and so far only, report on thin-section transmission electron microscopy of hepatocyte innervation was made by Yamada (45) who de-

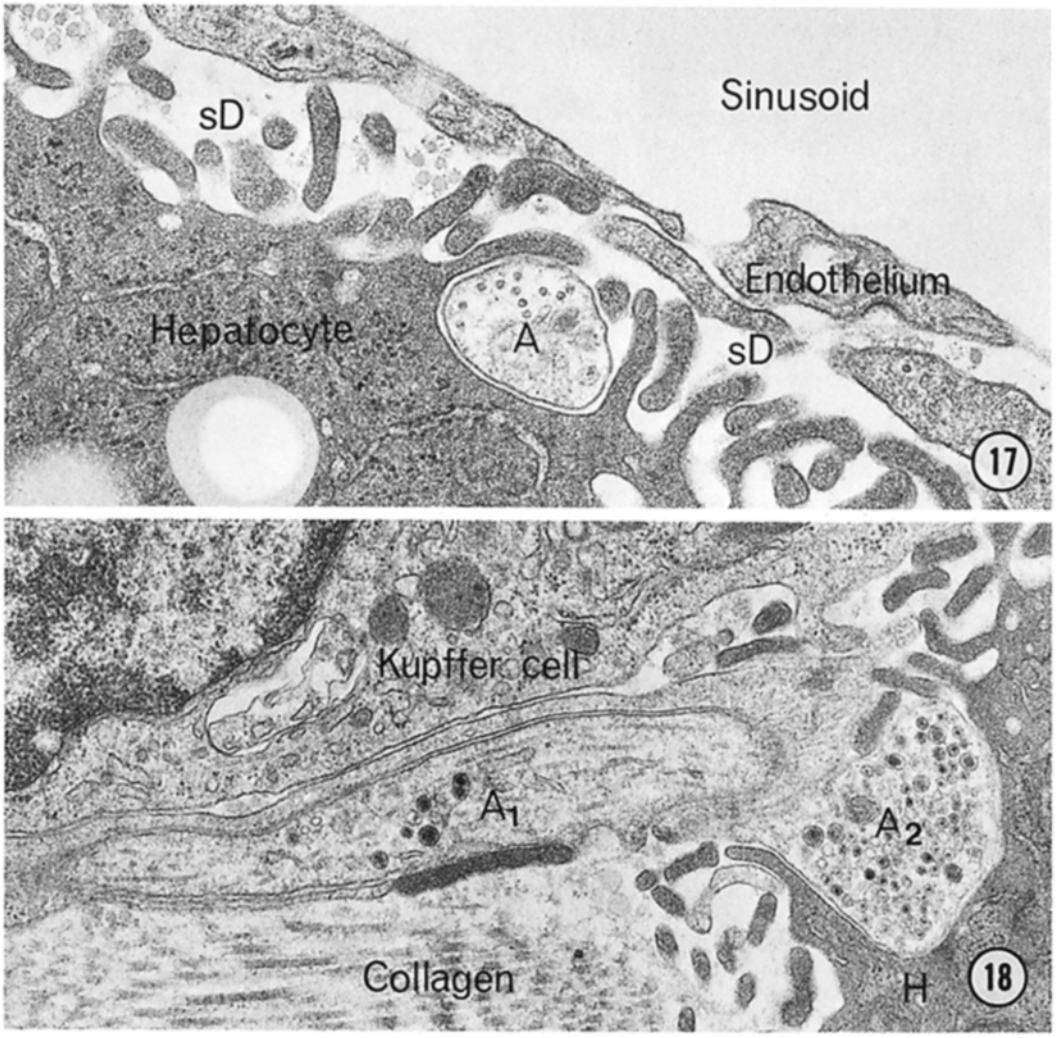

FIGURE 17 Transverse section of an intralobular axon (A) with microtubules almost surrounded by the hepatocyte after regeneration (same animal as in Fig. 18). Space of Disse (sD). × 31,500.

FIGURE 18 Liver nerve in regeneration phase after chemical denervation (same animal as in Fig. 18). One axon (A_1) is obliquely sectioned, and the terminal varicosity of another axon (A_2) adjacent to a hepatocyte is filled with dense-cored vesicles. × 21,500.

scribed nerve fiber-hepatocyte contacts in the mouse. In this species, he found that nerve fibers ended on the hepatocytes that were adjacent to the connective tissue of the triads. Although it seemed evident that these were efferent nerve fibers which contained transmitter vesicles characteristic of sympathetic nerves, the nature of these nerves was not determined. A more recent study (36) interpreted an extensive network of fibers in the rat liver lobules to be cholinergic nerves on the basis of their staining, fluorescence, and scanning electron microscopy. Contrary to these findings, all evidence in the present study indicates that intralobular nerves are norepinephrinergic.

Some recent studies on the presence and distribution of liver nerves in rat, cat, and dog, using fluorescence microscopy, have demonstrated liver innervation (3, 32, 35). With the use of similar fluorescence microscopy techniques, ligation of the guinea pig bile duct was reported to cause the degeneration of the liver nerves containing catecholamines and acetylcholinesterase (7, 41).

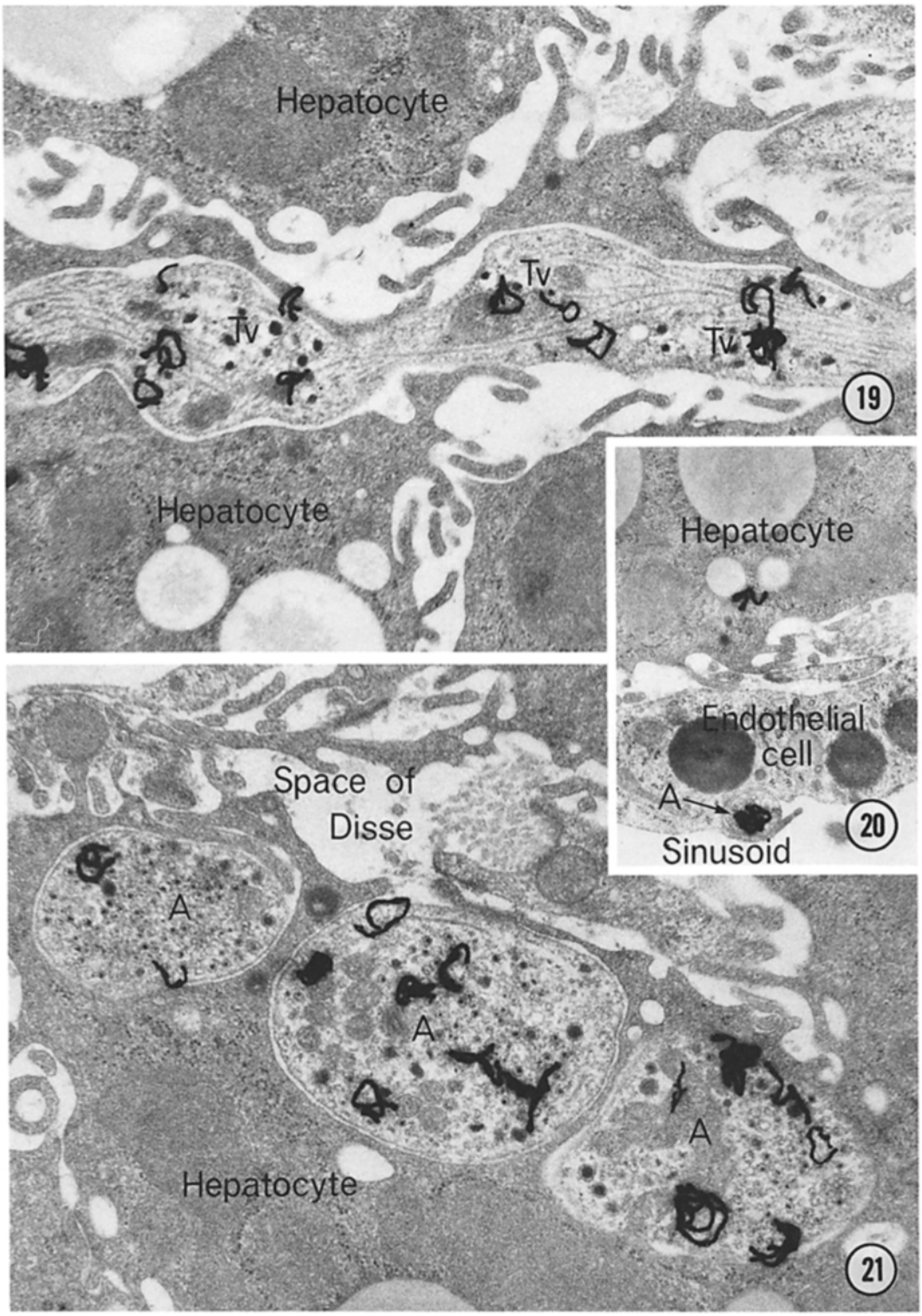

FIGURE 19 Autoradiograph of a longitudinal section through a liver nerve of *Tupaia* after injection with [^{3}H]norepinephrine (for details, see Materials and Methods). Note that the silver grains are preferentially localized to regions containing dense-cored vesicles (*Tv*). × 15,000.

FIGURE 20 A small axon autoradiographically labeled as in Fig. 23. This nerve runs along the endothelial cell on the luminal side adjacent to the sinusoid lumen. × 11,500.

FIGURE 21 Transverse section through intralobular nerves (*A*) containing many dense-cored transmitter vesicles. All nerves are labeled showing the uptake of exogenous norephinephrine. Autoradiography as in Fig. 18. × 16,000.

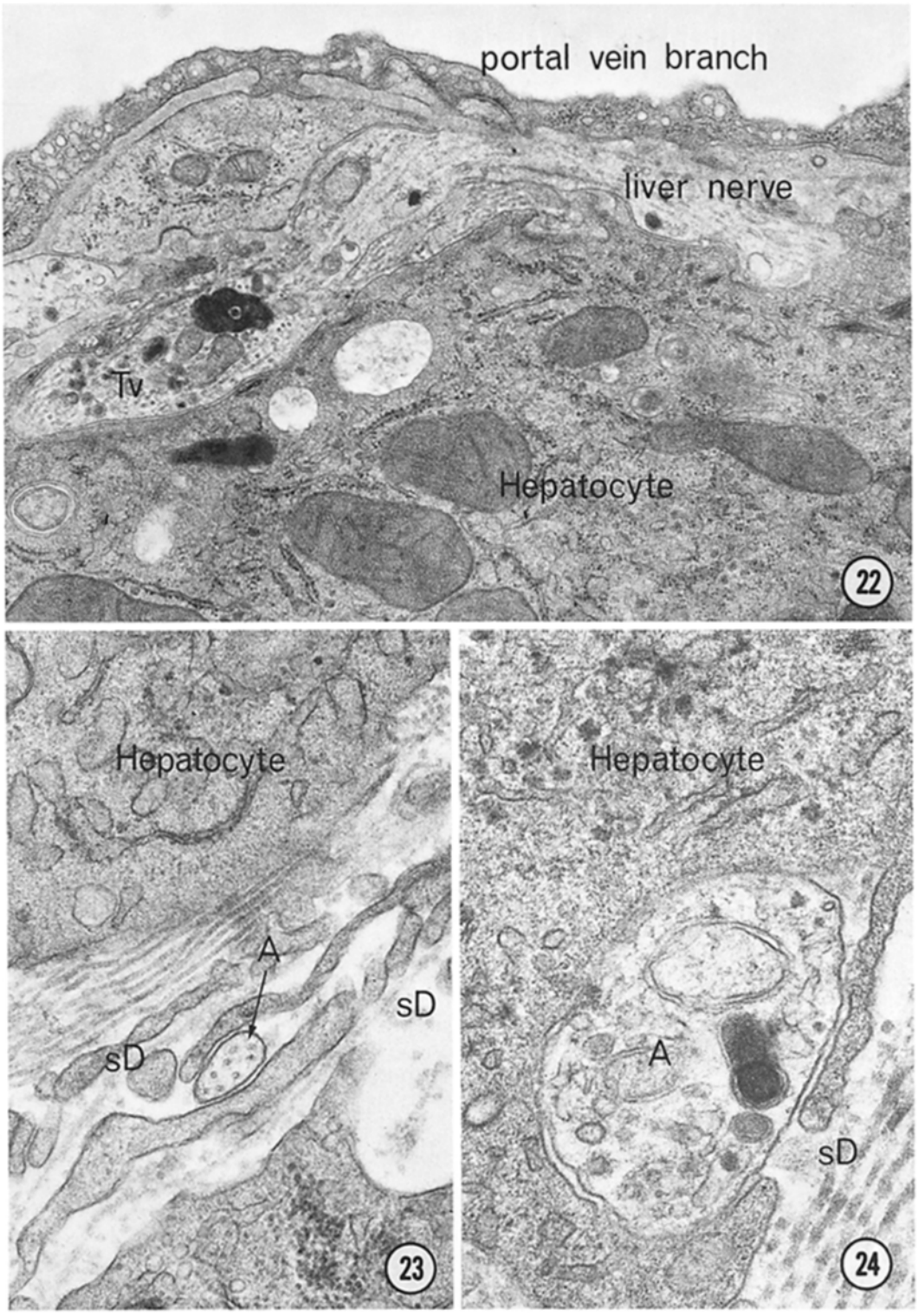

FIGURE 22 Liver nerve of *M. mulatta* in the region of a triad close to a branch of the portal vein. The nerve is in close contact with the hepatocyte and shows a varicosity with dense-cored transmitter vesicles. × 22,500.

FIGURE 23 A small profile of a liver nerve (*A*) in the space of Disse (*sD*) of *M. mulatta* in transverse section. Microtubules are present in the axon. × 33,500.

FIGURE 24 A terminal nerve fiber-hepatocyte contact of a nerve (*A*) deep in the hepatic lobule of *M. mulatta*. The nerve is almost surrounded by cytoplasmic extensions of the hepatocyte, and there is little contact with the space of Disse (*sD*). × 53,500.

These findings, however, have not yet been fully substantiated. The most conclusive investigations suggesting the role and complexity of liver innervation have been carried out by physiologists. Liver nerves may be classified into three physiological groups: (*a*) afferent nerves for chemoreceptors (29) and osmoreceptors (2), (*b*) efferent nerves for the vasomotor regulation of the liver blood flow (16), and (*c*) efferent nerves to the liver parenchyma for the elevation of glucose output (27). The importance of norepinephrine for liver function has also been emphasized by a study (18) which showed that this catecholamine administered to liver slices caused increased potassium efflux, hyperpolarization of the hepatocyte membrane, and an increase in membrane conductance.

In the present study, the autoradiographic localization of [H^3]norepinephrine and the characteristic fluorescence suggest that the *Tupaia* liver lobule is innervated exclusively by norepinephrine-storing endings and varicosities. This interpretation is substantiated further by chemical sympathectomy after 6-OH-dopamine injections (39, 40). Our results indicate that the liver parenchyma seems to be rather unique with exclusive efferent, catecholaminergic innervation. Since it has been shown that autonomic nerves take up different amounts of biogenic amines depending on the organs (38), it was surprising that the liver nerves were not labeled with other biogenic amines. However, some labeling of cardiac (11) and pancreatic (unpublished) nerves by [H^3]dopamine [H^3]epinephrine, and [5-H^3]hydroxytryptamine in the same animals used in the present investigation indicates the availability of these substances to the liver nerves.

However, many aspects of liver innervation remain to be clarified. There are no comprehensive studies of efferent liver nerve fibers, and the precise distribution of efferent nerves to elements of the triads remains to be elucidated. Furthermore, the apparent species differences in liver innervation are not understood. On the basis of the present observations, we have suggested that there may be an inverse relationship between electrotonic coupling, represented by gap junctions between liver cells, and the frequency of nerves in the hepatic lobule (12). It is interesting to note that in *Tupaia* the liver nerve fibers are most abundant in the periportal zone where gap junctions are quite rare. Some preliminary fluorescence microscopical observations have indicated that the liver of humans, guinea pigs, and cats have a nerve distribution similar to that of *Tupaia*. However, in the livers of mouse and rat there are few nerves that extend deep into the liver lobule.

Although we still do not know the functional relevance of the efferent innervation in the liver, we believe that it may be of importance in the regulation of liver metabolism by hormones such as insulin and glucagon. The extent to which liver function may be modulated, regulated, or controlled by these liver nerves remains to be established.

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