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Electron microscopic, histochemical and thin-layer chromatographic techniques have been used to study some cytological changes associated with seasonal lipid deposition in the liver of Ascaphus. Histochemical and chromatographic information has shown that unsaturated triglycerides occur in great quantities in the liver of preovulatory females and may serve as a solvent for estrogenic compounds as well as for carotenoids. This infiltration of hepatic cells is definitely sexually dimorphic and the livers of males show only a limited seasonal variability. A tri-laminate interface, of about the same dimensions as typical plasma membranes, was observed around the intracellular lipids. This interface suggests that

pinocytotic uptake augments the stores produced by intracellular lipogenesis proposed by other authors. The failure to observe colloidal lipid globules, chylomicra, anywhere in the liver suggests a subtle movement of lipids from extra hepatic reserves such as the skeletal muscle and other tissues. Preliminary electron microscopic and chromatographic data indicate a seasonal cessation of bile secretion during the winter fasting period. The sequence of morphological changes characteristic of the post-ovulatory rejuvenation phase further indicate that the rough vesicular endoplasmic reticulum represents a developmental stage in the formation of the compact tubular reticulum of the parenchymal cells.

THE DEPOSITION AND STORAGE OF HEPATIC LIPIDS IN THE FROG ASCAPHUS TRUEI (STEJNEGER): AN ELECTRON MICROSCOPIC, HISTOCHEMICAL AND CHROMATOGRAPHIC STUDY

by

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THE DEPOSITION AND STORAGE OF HEPATIC LIPIDS IN THE FROG ASCAPHUS TRUEI (STEJNEGER): AN ELECTRON MICROSCOPIC, HISTOCHEMICAL AND CHROMATOGRAPHIC STUDY

INTRODUCTION

Amphibian liver, as observed in light microscopy, is composed of numerous branching tubules within a reticular stroma. Under specific physiological conditions, the parenchymal cells comprising the walls of these tubules may accumulate lipid vesicles. The phenomenon of fatty infiltration in amphibian liver was known to early authors although they could not agree either on the time of its occurrence or its causes, nor did they explain the origin or types of lipid formed. Berg (1920, 1922 and 1924) studied the cyclic infiltration of lipids within the liver of a European salamander and noted that fats start to accumulate in the hepatic cells at the end of the winter fasting period. Ackerman (1949) used selective extraction methods to measure cyclic fluctuations in hepatic lipids and concluded that maximum fatty infiltration is observed in July and again in the winter months, corresponding with periods of maximum ovarian development. She also showed that triglycerides and cholesterol esters account for the major lipid fluctuations in Rana. Mukherji and Deb (1960) used histochemical methods to measure seasonal concentrations of lipids in the tissues of male Bufo and found that hepatic triglycerides become maximal during November whereas unsaturated

lipids and plasmalogens are most abundant in August.

It may be argued that extensive knowledge about all available energy stores must be obtained before the origin of hepatic lipids can be known. Berg (1920, 1922 and 1924) concluded that as salamanders do not feed during the hibernation period the fat must originate from other tissues and postulated a partial fatty metamorphosis of the skeletal muscles. It has been pointed out that Rana do not feed during the spawning period and therefore must draw on stored energy reserves from the onset of hibernation to post-ovulation (Smith, 1950). The resulting depletion of energy reserves is accelerated greatly by the almost complete utilization of the fat body and marked by the reduction of liver glycogen. Mizell (1965) found that blood sugar and liver glycogen levels are minimal in Rana at the conclusion of hibernation whereas the fat bodies continue to diminish until feeding is resumed, one month later.

There seem to be just three possible sources from which the frog might obtain lipids active in hepatic infiltration: 1) conduction or transformation of lipids from stored fat deposits, 2) intra-hepatic synthesis of fats from carbohydrates by the formation of long-chain fatty acids and subsequent esterification with glycerol to form triglycerides or 3) the "fatty metamorphosis" of other tissues such as skeletal muscle (Berg, 1924).

A relationship between fatty infiltration of the liver and

reproductive events was suggested by Berg and Falk (1924). Ackerman (1949) observed that fatty infiltration was much more pronounced in female Rana than in males and postulated that gonadotropic hormones might be dissolved in the hepatic lipids. However, her subsequent endocrinological tests gave no support to this theory (Ackerman, 1960). Mizell (1965), studying the cyclic energy reserves in Rana, made no distinction between the sexes other than to note that while spermatogenesis is completed by fall, oogenesis continues through winter.

Early studies with the light microscope left many morphological questions of hepatic lipid storage and transport unresolved until the advent of electron microscopy. The space of Disse has been shown to be a real perivascular space resulting from a peculiar relationship of the endothelial cells to the specialized surface of the hepatic parenchymal cells (Fawcett, 1964). Microvilli extend from the surface of the parenchymal cell into the space of Disse. This fluid-filled space must be traversed by all substances entering the parenchymal cells from the blood stream. Numerous investigators have injected rats with colloids and found that small droplets of esterified lipid known as chylomicra enter hepatic cells from the blood by means of pinocytosis (Ashworth, Stembridge, and Sanders, 1960; Parks, 1962; Edgren and Silversmit, 1963 and Robinson, 1964). Fawcett (1964, p. 212) however, casts some doubt on this observation by

the statement,

> While these are suggestive of pinocytosis there is in our experience, little evidence in the normal liver of uptake of lipid or other material in bulk from the space of Disse.

Deposits of intracellular lipid, as seen in electron micrographs, commonly occur in the form of intensely osmiophilic spherical droplets. This spherical shape has been used as a criterion of good fixation (Napolitano, 1963; Fawcett, 1966) even though these and other authors frequently publish electron micrographs showing lipid deposits with a crenated appearance (Napolitano, 1963; Napolitano and Gagne, 1963 and Fawcett, 1966).

Attempts have been made to verify a membrane system around intracellular lipid deposits (Sheldon and Angel, 1964). Such membranes have often been interpreted as components of endoplasmic reticulum associated with pinocytosis and transport of lipid droplets (Palay and Revel, 1964). Contrary to these reports Fawcett (1964) finds little evidence for any direct involvement of endoplasmic reticulum in lipid transport and he joins with other authors in characterizing these deposits as free within the cytoplasm, without a membrane (Wirsen, 1964; Fawcett, 1964).

Macroscopic observations made at the inception of this study led to the belief that Ascaphus truei would be a good experimental animal for histochemical and ultra-structural studies of hepatic lipids. Early in the course of this study it became evident that the

small quantity of liver tissue and the presence of other inseparable tissue components (Belton, 1962) made precise quantitative lipid measurements difficult. The present study is an endeavor to describe the cytology of hepatic lipid infiltration by correlating data of histochemical, electron microscopic and thin-layer chromatographic techniques.

METHODS AND MATERIALS

Thirty-two adult specimens of Ascaphus truei were collected during the pre-ovulatory and post-ovulatory period of spring (late April to middle of July). These animals were procured from two Oregon populations, Marys Peak in western Benton County and the South-fork of the McKenzie River in eastern Lane County. Animals were transported to the laboratory in a cold water bath to simulate their normal environmental temperature and reduce physiological changes in lipid metabolism. Other chemical and morphological changes such as those observed by Smith (1954) in Rana were minimized by fixing the livers immediately on arrival at the laboratory.

Pertinent information such as total animal weight, sex, wet weight and color of liver tissue was recorded. After this the liver was prepared for analysis by two or more techniques, thus facilitating the correlation of results from electron microscopy, histochemistry and thin-layer chromatography. The ovaries were removed from each female and preserved as an aid in staging the reproductive cycle.

Histochemistry

Tissue fixed in 10% calcium-formal solution was sectioned on the cryostat and stained with Nile blue sulfate to reveal neutral fats

according to the method of Cain as summarized by Barka and Anderson (1963). Frozen sections were affixed to albumenized glass slides and stained in a 1% Nile blue solution at 60° C. The sections were subsequently subjected to a short rinse in warm distilled water, a 30-second differentiation in 1% acetic acid and mounted in glycerin jelly. Adjacent sections of tissue were stained with a general lipid staining technique to verify lipid material since Barka and Anderson (1963) claim that a positive Nile blue sulfate test should not be used as exclusive evidence for lipids. Some frozen sections were treated in cold acetone containing magnesium chloride to remove the triglycerides, cholesterols, and sterols from the tissue prior to staining in Nile blue sulfate (Barka and Anderson, 1963).

Osmium tetroxide has been found to form diols and diesters when reacted with the monester bond of unsaturated lipids (Barka and Anderson, 1963). Adams (1960) showed that the osmiophilic staining reaction is dependent upon lipoidal ester bonds whereas it is independent of concentration of reducing groups of proteins and carbohydrates. From this rationale the following staining procedure was devised to localize and quantify the ethylene bonds within the hepatic lipids and to characterize the special distribution of lipid and glycogen stores.

1. 10 μ fresh frozen tissue sections applied to clean glass slides

2. fixed in 1% osmium tetroxide for 15 minutes (buffered to pH 7.3 with phosphate buffer)
3. hydrolized in 0.5% periodic acid for ten minutes
4. rinsed in two changes of distilled water
5. stained in Schiff's leuco-fuschin (15 minutes) at 39° C.
6. rinsed in three changes of bisulfite solution
7. dehydrated and mounted with synthetic mounting media

Phospholipids were demonstrated histochemically in tissue fixed in calcium-formal and post-fixed in dichromate according to the acid hematein method of Baker (1946). After remaining in 5% dichromate at 60° C for 24 hours, frozen sections were cut in the cryostat and floated on fresh dichromate solution for one hour. Henceforth, the sections were transferred from the surface of the following solutions with a three-millimeter nylon loop and according to the following schedule:

1. washed in distilled water for five minutes
2. five hours in acid hematein solution
3. differentiated and washed in borax ferricyanide solution (37° C) for 18 hours
4. placed in two changes of distilled water for ten minutes and mounted in glycerin jelly

Phospholipids and nucleoproteins were stained dark blue or blue-black while pyridine extracted control sections revealed very little

stain in non-lipid substances.

Thin-layer Chromatography

A minimum of one hundred milligrams of fresh liver tissue was fixed, emulsified and extracted in a one-to-one solution of methyl alcohol and chloroform according to Hanahan (1960). After being emulsified in the solvent the mixture was filtered, washed with water to remove soluble proteins and the purified lipid concentrated by evaporation. The total weight of extracted lipid was recorded. The extract was redissolved in chloroform and refrigerated.

Qualitative measurements were then made on the extracted lipids by thin layer chromatography (Mangold, 1961 and Rouser et al., 1964). About one-half microgram of extracted lipid was added to the bottom of a glass plate coated with silica gel G. Known standards were added to each plate to facilitate positive identification of lipid types and to aid in quantitative measurement. One-dimensional ascending chromatographs were produced with a solution of 90 parts hexane, ten parts ethyl ether and one part glacial acetic acid. The degree of bond saturation within the lipid samples was ascertained by exposing the plates to iodine vapors and outlining the resulting brown spots. The plates were then sprayed with H_2SO_4 solution and charred to reveal the identity and intensity of all lipid types.

The lipid classes separated in this manner were measured

semiquantitatively by use of a densitometer (Rouser et al., 1964). A Model 530 Photovolt densitometer equipped with a stage for 20 × 20 cm glass plates, and a Varicord recorder and integrator were used to make three density readings from which an average spot density was computed for each spot. A tracing of each spot was made on waxed paper and evaluation of spot size made from the paper weight. The percent of each lipid component was computed from the product of optical density times paper weight of the corresponding lipid spot. The assumption that the sum of all paper weights and densities originating from the same sample are equal to 100% of extracted lipid was validated by checking the values obtained for the standard lipids. These latter values fell within a 10% error.

Electron Microscopy

Electron microscopic observations were made on tissue sections fixed in cold 2% osmium tetroxide buffered to pH 7.3 with veronal acetate (Palade, 1952). Some tissue blocks were immersed in cold acetone containing magnesium chloride for 20 minutes prior to fixation to selectively extract glycerides, sterols, cholesterol and cholesterol esters without removing the phospholipids (Barka and Anderson, 1963).

Fixed tissue was prepared according to the following procedure. All tissue blocks were dehydrated and impregnated with Epon 812

polymerized according to Luft (1961). After sectioning on a Porter-Bloom microtome the sections were stained with saturated aqueous solution of uranyl acetate (Marinozzi and Gantier, 1962) with or without subsequent staining in lead hydroxide (Reynolds, 1963). Micrographs were taken on an RCA EMU 2D microscope at initial magnifications from 1, 200X to 18, 000X and photographically enlarged.

OBSERVATIONS

Macroscopic Observations

The liver of Ascaphus undergoes some seasonal changes associated with the reproductive cycle that can be noted macroscopically as differences in size, weight, color and consistency of the organ. At the conclusion of hibernation the liver of female frogs shows an accumulation of carotene pigments and a two fold increase in size and weight. These changes seem to be associated with preparation for the ensuing reproductive events. During this pre-ovulatory period the liver of the sexually mature female assumes a bright yellow color and soft consistency whereas the liver retains a deep brown color in the male frog. This sexual dimorphism is lost just prior to ovulation as the lipids are mobilized from the female liver.

Ovarian size and development was used as an index to correlate changes in liver physiology with reproductive events (Plate I). The terms small, medium and giant will be used to describe ovarian size.

Histochemical Observations

Histochemical methods were used to characterize most of the lipids responsible for the fatty infiltration and to identify any cellular

localization or specialization associated with this lipid storage. These observations are based upon tests made with four males and eight females, including two immature, three with medium ovaries and three with giant ovaries. The pink reaction produced by Nile blue sulfate helped to establish that neutral fats play an important role in the seasonal infiltration of the female Ascaphus liver (Plate II, fig. 1). Further verification of involvement of one or more neutral fats was obtained when cold acetone pre-treatment was found to negate the subsequent staining reaction of Nile blue sulfate. The tissue was left with a spongy appearance due to the resulting extraction of the neutral fats (Plate II, fig. 2). Hepatic tissues from immature females or males failed to show any histochemical reaction for neutral fat and presented a deep blue color indicative of a compact tissue organization (Plate II, fig. 3).

Liver sections were fixed in 1% osmium tetroxide to visualize the distribution of lipid, followed by staining with the periodic acid-Schiff reaction to demonstrate the glycogen stores within the liver (Plate II, fig. 4). It was found that both fats and glycogen occur within the parenchymal cells concurrently (Plate II, fig. 4). The osmophilic reaction was of equal intensity in all lipid vesicles, suggesting that all infiltrating lipids have an equal number of ethylene bonds or that saturated and unsaturated lipids are uniformly intermixed within the vesicles.

Changes in the localization and concentration of hepatic phospholipids could not be shown by means of the acid hematein stain. Therefore no evidence was found for Ackerman's suggestion (1949) that phospholipids play an important role in fatty infiltration of the liver.

Thin-layer Chromatography

Thin-layer chromatography afforded more precise characterization of hepatic lipids and led to a semi-quantitative measurement of the fats responsible for the pre-ovulatory infiltration. Four classes of lipoidal compounds: 1) phospholipids, 2) free sterols, 3) triglycerides 4) bound sterols in association with carotene, were always found in the hepatic extracts. High molecular weight waxes were occasionally found in small quantities (Table 1 animal number 29 and 32). The triglyceride and bound sterol concentrations showed the greatest fluctuation during pre-ovulatory hepatic infiltration (Plate III). The stippled borders surrounding chromatographic spots (Plate III) mark the occurrence of unsaturated bonds as revealed by iodine vapors. It was noted that with the exception of free sterols and waxes all lipid components demonstrate some degree of unsaturation; the greatest concentration of ethylene bonds being found in the triglycerides of the pre-ovulatory female.

Electron Microscopy

The ultrastructure of the Ascaphus liver does not differ markedly from the description of Rana liver by Fawcett (1955, 1964). The parenchymal cells are arranged in bi- or tri-layered lamina and are set apart from the circulatory system by the perivascular space of Disse through which all substances entering or leaving the hepatic circulation must pass. The hepatic stroma has as its two most common constituents melanocytes and fibroblasts. These can frequently be seen in sections (Plate XII).

The surface of the parenchymal cells adjacent to the sinusoids takes the form of numerous irregularly oriented microvilli (Plate IV). The parenchymal cells are firmly held one to another by very tight interlocking cellular processes (Plates XI and XIII). Bile capillaries occur at the point of convergence of four or five parenchymal cells (Plates VI, X and XV). The canaliculus is a tubular structure into which many microvilli from the parenchymal cell surfaces project (Plate XV). Depending on the animal's physiological state, the cytoplasmic matrix may show varying quantities of glycogen granules, mitochondria, osmophilic dense bodies, endoplasmic reticulum and lipid droplets.

In order to better understand the sequence of ultrastructural changes taking place within the hepatic cells of the frog, four

physiological groups were examined. The male frog formed a unit by itself while the female was subdivided into one of the following three groups: 1) pre-ovulatory with maximum lipid infiltration 2) pre-ovulatory with decreased lipid storage resulting from the mobilization of fats 3) post-ovulatory with changes that account for cellular reorganization.

The hepatic cells of the male Ascaphus are very electron dense, the parenchymal matrix appearing filled to capacity with osmophilic inclusions (Plates IV and V). There are a limited number of lipid droplets and small dense bodies that resemble lysosomes. The greatest portion of the cytoplasm is occupied by a compact mixture of glycogen, rough tubular endoplasmic reticulum and small dense mitochondria (Plates IV and V).

Female frogs collected early in the spring (about two months prior to ovulation and having an ovarian development similar to animal number 23, Plate I) already showed morphological changes associated with hepatic lipid infiltration. The lipid droplets are homogeneous, dense, usually spherical structures and are clumped within the cytoplasm in close association with the mitochondria and glycogen. The endoplasmic reticulum which was without exception of the rough type, was displaced into densely packed regions of irregular contour (Plates VI, VII and VIII). Some parenchymal cells having reduced lipid inclusions were found to lack the usual

tubular endoplasmic reticulum and possessed a distinctive reticulum of vesicular form (the lower cell in Plates VII and VIII).

Electron micrographs made of tissues selectively extracted with acetone gave verification that the major portion of the lipid droplets were neutral fats. Small quantities of unextracted lipid residue suggested that neutral fats function as an intracellular solvent for other lipid types (Plate IX). The small dense bodies identical to those found in the male were resistant to acetone extraction (Plate IX).

Females possessing fully developed ovaries (animal number 30, Plate I) showed a very distinct change in hepatic cell morphology just prior to ovulation. As the lipids are moved out of the cells relatively large areas are left devoid of ordered cellular structure (Plates X and XI). The small dense mitochondria which were once in close association with the lipid droplets become very prominent within a relatively structureless cytoplasm. Only a poorly developed endoplasmic reticulum remains (Plate X). As the lipid droplets are reduced a bilaminate membrane-like interface appears around the remaining fat (Plates X and XI). The resulting vesicle is irregularly shaped and partially filled with fat. The regions of tubular endoplasmic reticulum usually occur at the periphery of the fat depleted parenchymal cell (Plates X and XI).

The first morphological evidence of post-ovulatory hepatic

rejuvenation is the proliferation of a vesicular type of rough endoplasmic reticulum (Plates XII, XIII, XIV and XV). The area immediately surrounding the sparse irregularly shaped lipid droplets is the last to be filled in by the regenerating reticulum (Plates XII and XIII). The final stages of reticular generation are characterized by a uniform dispersal of mitochondria, dense bodies and an interspersion of vesicular endoplasmic reticulum and irregular regions of tubular endoplasmic reticulum (Plate XV). Small electron transparent bodies of unknown origin were noted in tissue sections at this stage.

DISCUSSION

The knowledge of seasonal lipid storage within the frog liver is not new but is very deserving of renewed investigations relating to the physiology of the total animal. It has become very clear, in the course of this study, that morphological and physiological factors related to hepatic lipid infiltration cannot be understood without extensive knowledge of the concurrent changes taking place in the ovaries (Ackerman, 1949 and Smith, 1950), the thyroid (Smith, 1954, and the adrenal glands (Smith, 1954). Similarly relationship to other energy stores in the blood and fat bodies must be considered. It is very regrettable that many studies on cyclic energy changes in amphibians have been based exclusively on seasonal designations without fully considering all of the parameters such as environmental temperature, feeding habits and reproductive factors. A conscious effort has been made to relate the morphological and histological findings of this study to known physiological changes. These efforts have not been totally satisfactory for there are many physiological questions concerning Ascaphus for which little or no information is available. For instance, nothing is known about the extent of hibernation, the winter feeding habits or to what degree these factors influence lipid metabolism. Mizell (1965) found reason to believe that nutritional factors did not affect seasonal changes in energy reserves

in Rana. This rationale was not followed in the present investigation even though it is believed that the low water temperature characteristic of the Ascaphus environment (about 4° C.) might retard and reduce any effects caused by fasting.

Two types of fatty liver conditions are recognized which have as their basis the level of triglyceride concentration in the plasma (Robinson, 1964). It has been demonstrated that poisoning by administration of ethionine, carbon tetrachloride or white phosphorus results in fatty liver due to the blockage of triglyceride release. This release of triglycerides into the plasma is dependent upon hepatic synthesis of the protein moiety of a low density plasma lipoprotein (Robinson, 1964; Eder, et al., 1964). Injections of noradrenaline and of certain anterior pituitary hormones also result in the development of fatty livers, but these are associated with high levels of triglyceride already present in the plasma (Robinson, 1964). The nature of the seasonal hepatic infiltration of female frogs has not been disclosed.

Numerous observations that the onset of hepatic lipid infiltration occurs during the middle of an extended fasting period (Berg, 1920, 1922, and 1924; Smith, 1950) have led investigators to propose that these lipids do not arise from nutritional sources. The suggestion by Berg (1924) that fats must originate from partial fatty metamorphosis of the skeletal muscle does not seem plausible in light of

our present knowledge about physiological equilibria. From the inception of this study two other possible sources for the lipid have been considered: 1) conduction and transformation of known fat deposits and 2) intrahepatic synthesis of fats from carbohydrates through glycolysis, formation of long-chain fatty acids and subsequent esterification with glycerol to form triglycerides. Smith (1950) found that the fat bodies of female Rana are reduced in size more rapidly than those of the male during the latter part of hibernation, indicating a possible lipid mobilization from the fat bodies to the hepatic cells. Failure to observe lipid transport bodies, chylomicra, in the liver either during the infiltration or mobilization phase prevented the visualization of morphological mechanisms of lipid movement into the parenchymal cells although these bodies have been observed by Trotter (1964) in regenerating rat liver. Preliminary histochemical tests for plasmalogens in Ascaphus liver agreed with the negative results of Mukherji and Deb (1960) in Bufo and thus gave no support to the theory of lipid formation via carbohydrate breakdown. Further physiological experiments based upon the labeling of lipid stores or seasonal changes in the concentration of lipids in the plasma may help identify the source of lipids responsible for liver infiltration.

If indeed lipids are removed from the fat bodies to infiltrate the liver for a brief time prior to ovulation, their function in the liver is not known. Ackerman (1960) attempted to demonstrate that

sex hormones are dissolved in the neutral fat portion, but her endocrinological tests were negative. The present study showed that when an excessive amount of extracted lipid sample was applied to thin-layer chromatographic plates and charred in the usual manner, a sex hormone component was found to exist proportional to the triglyceride concentration. This steroid hormone could be distinguished from the cholesterol fraction by the characteristic light blue color it assumed during the early part of the charring process. In light of this preliminary identification of a hormone component within the cholesterol fraction from female Ascaphus, more sensitive tests should be used to characterize its effect.

Additional information about the quantities of each lipid component must be gathered before a precise evaluation can be made. The hepatic triglyceride concentration undergoes a two-fold increase early in the period of ovarian development (Table 1) whereas the fluctuations noted during the terminal stages of ovarian development are indicative of the sudden and rapid mobilization of lipids from the liver.

Despite numerous electron microscopic reports to the contrary, most lipid droplets seen in liver cells lie free within the cytoplasmic matrix and are devoid of enclosing membranes (Fawcett, 1964).

> Most of these reports can be attributed to misinterpretation of a common artifact of osmium fixation in which the periphery of a lipid droplet stains more heavily than the

Table 1. Quantities of major lipid components extracted from Ascaphus liver.

Animal number	Sex & gonad size	"Fresh" wt. of liver	Weight of lipid	% Lipid content	Phospholipid	Free sterol	Triglyceride	Bound sterol	Waxes
20 & 26	♂	*217 mg.	17.6 mg.	8.1%	23.0%	18.8%	30.4%	27.6%	0
					21.3%	18.0%	29.6%	30.8%	0
21	♂	*209 mg.	14.4 mg.	6.9%	22.5%	16.3%	33.8%	26.8%	0
					22.9%	15.5%	35.0%	26.5%	0
22	♂	*207 mg.	16.2 mg.	7.9%	18.1%	14.2%	39.0%	28.6%	0
					17.9%	15.3%	38.8%	28.0%	0
32	♂	116 mg.	13.5 mg.	11.5%	17.2%	13.5%	46.5%	32.6%	trace
					18.1%	13.2%	35.8%	32.8%	trace
23 & 25	♀-small	*497 mg.	60.3 mg.	12.1%	10.8%	15.7%	37.6%	35.8%	0
					11.2%	17.1%	35.2%	36.2%	0
28	♀-medium	451 mg.	42.4 mg.	9.4%	7.6%	5.6%	76.2%	10.3%	trace
					2.1%	1.6%	90.5%	5.9%	trace
31	♀-medium	288 mg.	26.5 mg.	9.2%	13.5%	trace	80.0%	6.5%	0
					5.5%	trace	86.0%	8.5%	0
24	♀-giant	*301 mg.	22.8 mg.	7.6%	17.8%	17.4%	45.7%	19.0%	0
					18.0%	18.2%	44.5%	19.1%	0
27	♀-giant	305 mg.	21.9 mg.	7.2%	19.3%	4.5%	64.6%	11.1%	trace
					17.9%	7.9%	61.8%	12.3%	trace
29	♀-giant	350 mg.	24.5 mg.	7.0%	18.1%	14.0%	34.2%	33.6%	sizable
					19.3%	11.2%	34.6%	34.6%	sizable
30	♀-giant	240 mg.	22.7 mg.	9.5%	17.5%	trace	37.4%	45.0%	0
					17.5%	trace	51.5%	31.0%	0

* Weight does not include small quantity of tissue used for electron microscopy or histochemistry.

> interior, or else osmium diffuses from an intensely stained droplet into the adjacent cytoplasm producing a dense rim that can be mistaken in electron micrographs for a membrane. (Fawcett, loc. cit., p. 213).

However newly assimilated lipid droplets are often found within the membrane-limited channels of endoplasmic reticulum (Palay, 1964). Fawcett (loc. cit.) on the other hand, thinks that the presence of a limiting membrane around inclusions is suggestive of pinocytotic uptake. Electron micrographs made in the course of this study also clearly demonstrate a membrane-like interface around the shrinking lipid droplets characteristic of the mobilization phase just prior to ovulation (Plates X and XI). This interface is composed of a trilamellar structure approximately two times the thickness of a unit membrane. It is believed that the densely compact nature of the tissue prevents the clear recognition of the interfaces around lipid droplets during the period of infiltration.

The changes in the structure of the cell associated with the assimilation and subsequent release of lipid provide bases for some interesting speculations. Fawcett (1964) reviewed the functions historically attributed to the hepatic agranular endoplasmic reticulum. On the basis of isolation and chemical characterization, he discredited the earlier suggestion that the smooth endoplasmic reticulum is involved in glycogen metabolism and made a counterproposal associating this membrane structure with bile

secretion. The following data though fragmentary are indicative of seasonal interruption of bile secretion during the pre-ovulatory period: 1) stomach content analysis shows that Ascaphus does not feed during the pre-ovulatory period, thus making the production of bile salts unnecessary 2) preliminary attempts to demonstrate bile salts histochemically were negative and 3) no smooth endoplasmic reticulum was found in the course of this study. Further investigation in the feeding habits and seasonal measurements of secretory activity may indeed show that bile production ceases during the winter.

The replacement of vesicular endoplasmic reticulum subsequent to lipid mobilization is somewhat reminiscent of observations made on hepatic cells from starved rats (Fawcett, 1955). Berg's observation (1920) that the amount of basophilic material in the hepatic cytoplasm diminishes concurrently with the winter fasting period was supplemented by electron microscope studies (Fawcett, 1955). It was shown that a densely packed tubular endoplasmic reticulum, the counterpart to the basophilic bodies, underwent a significant decrease and dispersion during the period of prolonged fasting. In rats this period is marked by an increased lipid deposition. The condensation and displacement of the tubular endoplasmic reticulum noted in fasting Ascaphus is difficult to reconcile with the proposed diminution or dispersion (Plates VI and VIII) of this entity. The apparent reduction in basophilic material during this period of

increase may be attributed to an increased cellular volume, hence a reduced concentration of basophilic material.

It is premature to give a functional interpretation to the role played by the vesicular endoplasmic reticulum in the post-ovulatory rejuvenation phase. The observed morphological changes lead one to postulate a cyclic lipid deposition within the hepatic cells. It is possible that the vesicular endoplasmic reticulum coalesces to form the tubular endoplasmic reticulum during this cycle. If this explanation is correct, the occasional parenchymal cells containing only vesicular reticulum may represent an incompletely differentiated cell.

The dense bodies found with equal regularity in the hepatic cells of both sexes (Plates V, IX, X and XI) are similar to the structures identified as lysosomes (Fawcett, 1966). The positive identification can not be made on the basis of this study even though their resistance to acetone extraction rules out any association with neutral fats.

SUMMARY

Lipid infiltration and storage occurs normally within the hepatic cells of the hibernating frog. The degree and types of lipid stored are apparently related to numerous other interrelating physiological conditions. The aim of the present investigation has been to use morphological, histochemical and chromatographic approaches to study some principal problems in this process and to provide a structural basis for the interpretation of biochemical and physiological findings.

Unsaturated triglycerides account for the major portion of the lipids infiltrating the liver of female Ascaphus. Hepatic triglycerides occurring in great quantity might serve as solvents for estrogenic compounds as well as for carotenoids.

The increase of hepatic lipids in the female frog is not derived from a direct nutritional intake but originates from some internal reserve. Implication of three possible sources has been considered: 1) fatty metamorphosis of skeletal muscle, 2) intra-hepatic lipogenesis from carbohydrates or 3) conversion and transport of lipids from other tissues.

A tri-lamellar interface around the intracellular lipids may be indicative of a pinocytotic uptake and may rule out any intra-hepatic lipogenesis. This membrane-like structure has about the same

dimensions as other cellular membranes.

Failure to identify smooth endoplasmic reticulum in electron micrographs of parenchymal cells suggests that the bile secretory activity is interrupted during the winter fasting period.

The sequence of morphological changes characteristic of the post-ovulatory rejuvenation phase suggests that the rough vesicular endoplasmic reticulum may represent a developmental stage in the formation of the tubular type of reticulum.

BIBLIOGRAPHY

Ackerman, J. 1949. The annual rhythm of the fatty metamorphosis of the liver in the frog, Rana esculenta L. Bulletin International de l'Académie des Sciences de Cracovie, Ser. B pt. II, p. 145-174.

Ackerman, J. 1960. Rytm roczny stuszczén watroby zaby wodnej (Rana esculenta L.). Folia Morphologica 11:257-260.

Ashworth, C. T., V. A. Stembridge and E. Sanders. 1960. Lipid absorption, transport and hepatic assimilation studied with electron microscopy. American Journal of Physiology 198: 1326-1328.

Baker, John R. 1946. The histochemical recognition of lipine. Quarterly Journal of Microscopical Science 87:441-470.

Barka, Tibor and Paul J. Anderson. 1963. Histochemistry: Theory practice and bibliography. New York Harper and Row. 660 p.

Belton, John C. 1961. A histological study of the digestive system of Ascaphus truei (Stejneger). Master's thesis. Corvallis, Oregon State University. 51 numb. leaves.

Berg, W. 1920. Über functionelle Liberzellstrukturen I. Die Liberzelle von Salamandra maculata während des zustandes der guten Einwährung und des Hungers. Die Einwirkung von Futterung und von Beförderung der Gallenabsonderung bei Hungertieren. Archives für mikroskopische Anatomie und Entwicklungsmechanik 94: 234-251.

Berg, W. 1922. Über functionelle Liberzellstrukturen II. Das Verhalten des Fettes in der Liber von Salamandra maculata unter verschiedenen Bedingungen der Jahreszeit und der Ernährung. Archives fur mikroskopische Anatomie und Entwicklungsmechanik 96: 54-76.

Berg, W. 1924. Über functionelle Liberzellstrukturen III. Periodische Veränderungen in Fattgehalt der Leberzellen des im Winter hungernden Salamanders und ihre Ursachen. Zeitschrift fur Mikroskopisch Anatomische Forschung 1:245-296.

Berg, W. and V. Falk, 1924. Mikroskopische Untersuchungen uber den Zusammenhang von Eiweissabbau und Fettgehalt in den quergestreiften Muskelfasern des Frosches im Winter. Zeitschrift fur Mikroskopisch Anatomische Forschung 1:297-309.

Eder, Howard A., Paul S. Roheim, Lewis I. Gidez and Sam Switzer. 1946. Regulation of lipid transport in the liver. In: Proceedings of an International Symposium on Lipid Transport, Nashville, 1963, ed. by H. C. Meng. Springfield, Illinois, Thomas. p. 202-209.

Edgren, Bo and D. B. Zilversmit. 1963. Uptake of doubly labeled chylomicrons by dog liver slices and adipose tissue. Federation Proceedings 22:517.

Fawcett, D. W. 1955. Observations on the cytology and electron microscopy of hepatic cells. Journal of National Cancer Institute 15:1475-1503.

Fawcett, Don W. 1964. Morphological consideration of lipid transport in the liver. In: Proceedings of an International Symposium on Lipid Transport, Nashville, 1963, ed. by H. C. Meng. Springfield, Illinois, Thomas. p. 210-222.

Fawcett, Don W. 1966 . An atlas of fine structure: The cell, its organelles and inclusions. Philadelphia, W. B. Saunders. 448 p.

Hanahan, Donald J. 1960. Lipide chemistry. New York, Wiley. 325 p.

Luft, John H. 1961. Improvements in epoxy resin. Journal of Biophysical and Biochemical Cytology 9:409-414.

Mangold, Helmut K. 1961. Thin-layer chromatography of lipids. Journal of American Oil Chemists Society 38:708-727.

Marinozzi, V. and A. Gautier. 1962. Fixations et colorations: Étude des affinitiés des composants nucléoprotéiniques pour l'hydroxyde de plomb et l'acetate d'uranyle. Journal of Ultrastructure Research 7:436-451.

Mizell, Sherwin. 1965. Seasonal changes in energy reserves in the common frog, Rana pipiens. Journal of Cellular and Comparative Physiology 66:251-258.

Mukherji, Mrityunjoy and Chandicharan Deb. 1960. Lipid distribution in the tissues of hibernating and non-hibernating toad (Bufo melanostictus)--A histochemical study I. sudanophilia, unsaturated lipid and plasmalogen. Journal of Histochemistry and Cytochemistry 8:242-247.

Napolitano, L. 1963. The differentiation of white adipose cells: An electron microscopic study. Journal of Cell Biology 18: 663-679.

Napolitano, L. and Harriet T. Gagne. 1963. Lipid-depleted white adipose cells: An electron microscopic study. Anatomical Record 147:273-294.

Palade, G. E. 1952. A study of fixation for electron microscopy. Journal of Experimental Medicine 95:285-297.

Palay, Sanford L. and Jean Paul Revel. 1964. The morphology of fat absorption. In: Proceedings of an International Symposium on Lipid Transport, Nashville, 1963, ed. by H. C. Meng. Springfield, Illinois, Thomas. p. 155-170.

Parks, H. F. 1962. On the uptake of chylomicrons by hepatic cells of mice. Anatomical Record 142:320.

Revel, Jean Paul. 1964. Electron microscopy of glycogen. Journal of Histochemistry and Cytochemistry 12:104-114.

Reynolds, Edward S. 1963. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. Journal of Cell Biology 17:208-213.

Robinson, D. S. 1964. The uptake and release of lipids by the liver. In: Proceedings of an International Symposium on Lipid Transport, Nashville, 1963, ed. by H. C. Meng. Springfield, Illinois, Thomas. p. 194-201.

Rouser, George et al. 1964. Analytical fractionation of complex lipid mixtures: DEAE cellulose column chromatography combined with quantitative thin layer chromatography. Journal of American Oil Chemists Society 41:836-840.

Sabatini, D. D., K. Bensch and R. J. Barrnett. 1962. New means of fixation for electron microscopy and histochemistry. Anatomical Record 142:274.

Sheldon, Huntington and Aubie Angel. 1964. Some considerations on the morphology of adipose tissue. In: Proceedings of an International Symposium on Lipid Transport, Nashville, 1963, ed. by H. C. Meng. Springfield, Illinois, Thomas. p. 155-170.

Smith, C. L. 1950. Seasonal changes in blood sugar, fat body, liver glycogen and gonads in the common frog, Rana temporaria. Journal of Experimental Biology 26:412-429.

Smith, C. L. 1954. The relation between seasonal hyperglycemic and thyroid activity in the frog, Rana temporaria. Journal of Endocrinology 10:184-191.

Trotter, Nancy L. 1964. A fine structure study of lipid in mouse liver regenerating after partial hepatectomy. Journal of Cell Biology 21:233-244.

Wirson, Class. 1964. Studies in lipid metabolism. Acta Physiologica Scandinavica 65 (suppl. 252):1-39.

APPENDIX

Plate I

One ovary from each female frog is shown for the approximate staging of reproductive events. The word descriptions used for the ovarian sizes were small - - - - - - - 23 and 25

medium - - - - - 28 and 31

giant - - - - - - - 24, 27, 29 and 30

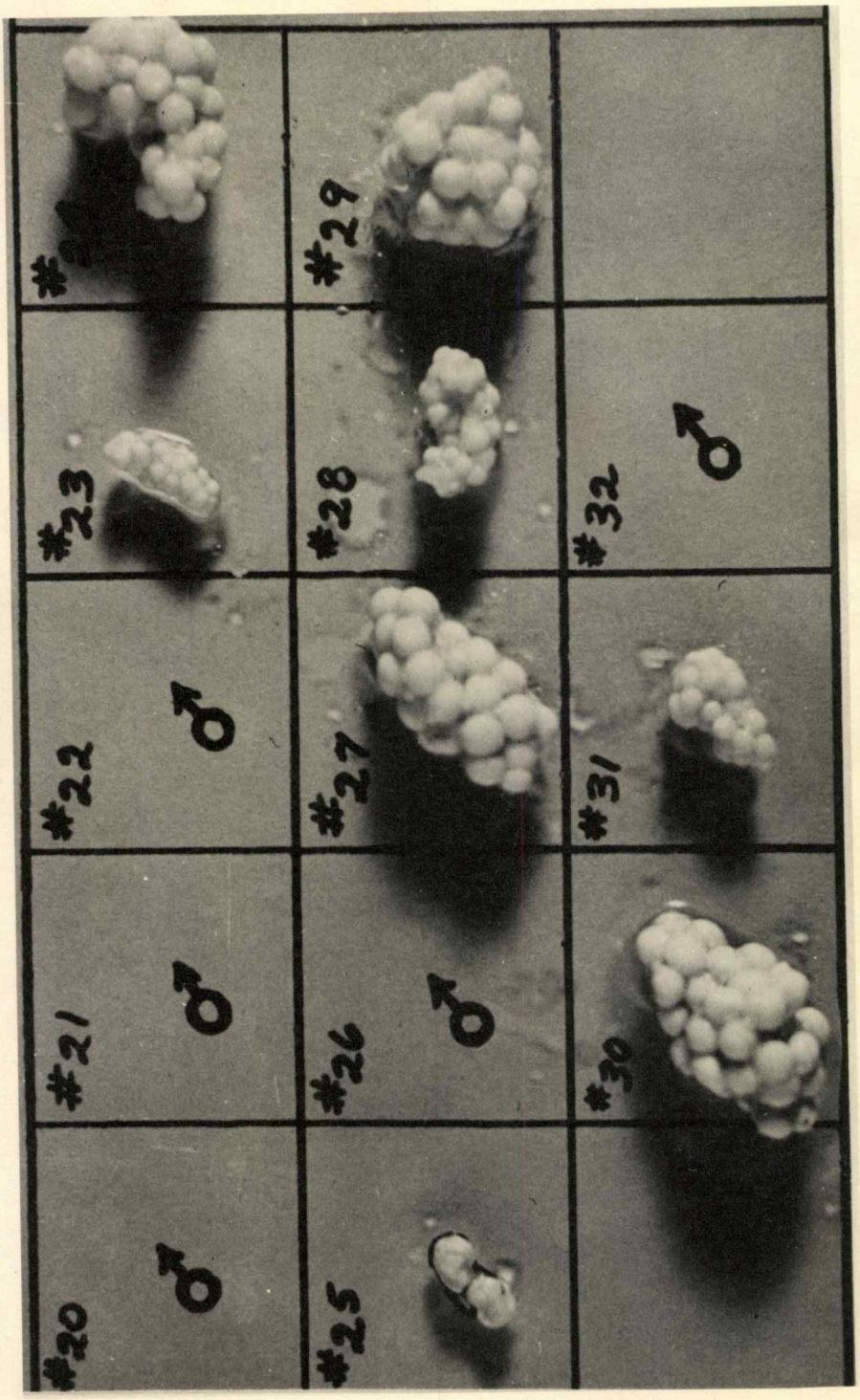

#20
♂
#21
♂
#22
♂
#23
#25
#26
♂
#27
#28
#29
#30
#31
#32
♂

Plate II

fig. 1-3

Nile blue sulfate reaction for neutral fat in the hepatic cell of Ascaphus

fig. 1 Pre-ovulatory female. The positive pink reaction shows a uniform distribution throughout the field.

fig. 2 Pre-ovulatory female. After extraction of neutral fats by cold acetone treatment.

fig. 3 Male. Liver showing no deposition of neutral fats

fig. 4 Unsaturated bonds of lipids demonstrated in hepatic tissue of a pre-ovulatory female by fixation with osmium tetroxide (brown color). Liver glycogen has been stained red by the periodic acid-Schiff reaction.

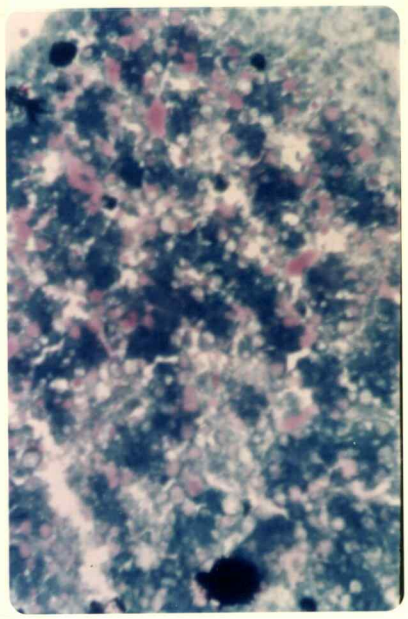


fig. 1

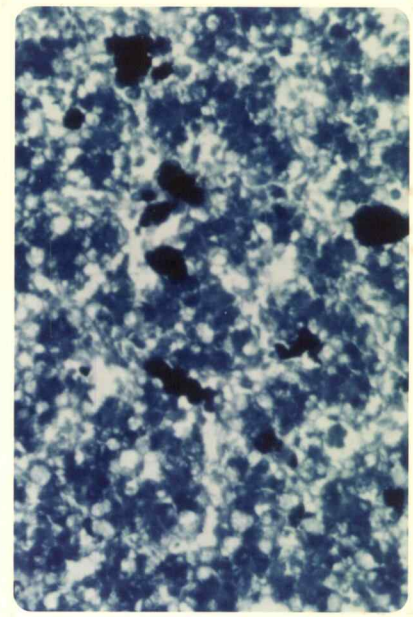


fig. 2

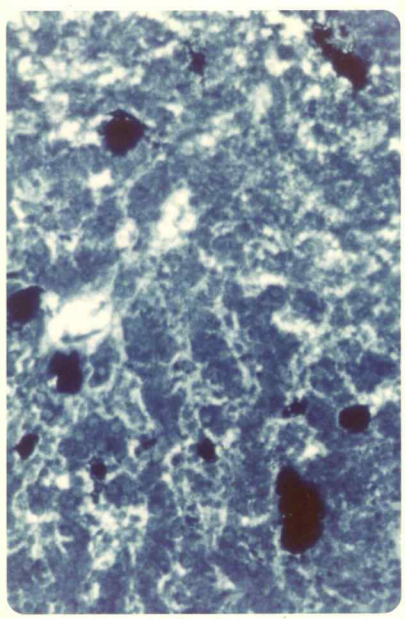


fig. 3

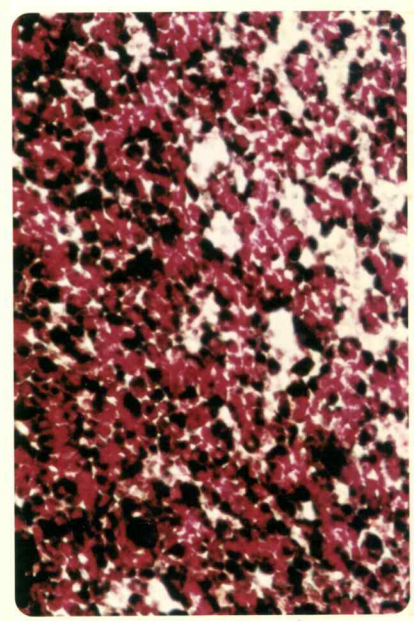


fig. 4

Plate III

Duplicate Silica Gel G chromatograms--

Eluting solution: 90 parts hexane, ten parts ethyl ether and one part glacial acetic acid. From the origin on the extreme right reading to the left are 1. polarized phospholipids

2. free sterols
3. triglycerides (constituting the most variable components)
4. a mixture of carotenes, bound sterols and sex hormones

Stippled borders indicate the presence of saturated ester bonds. High molecular weight waxes are seen to the left of the tryglyceride fraction in samples 29 and 32.

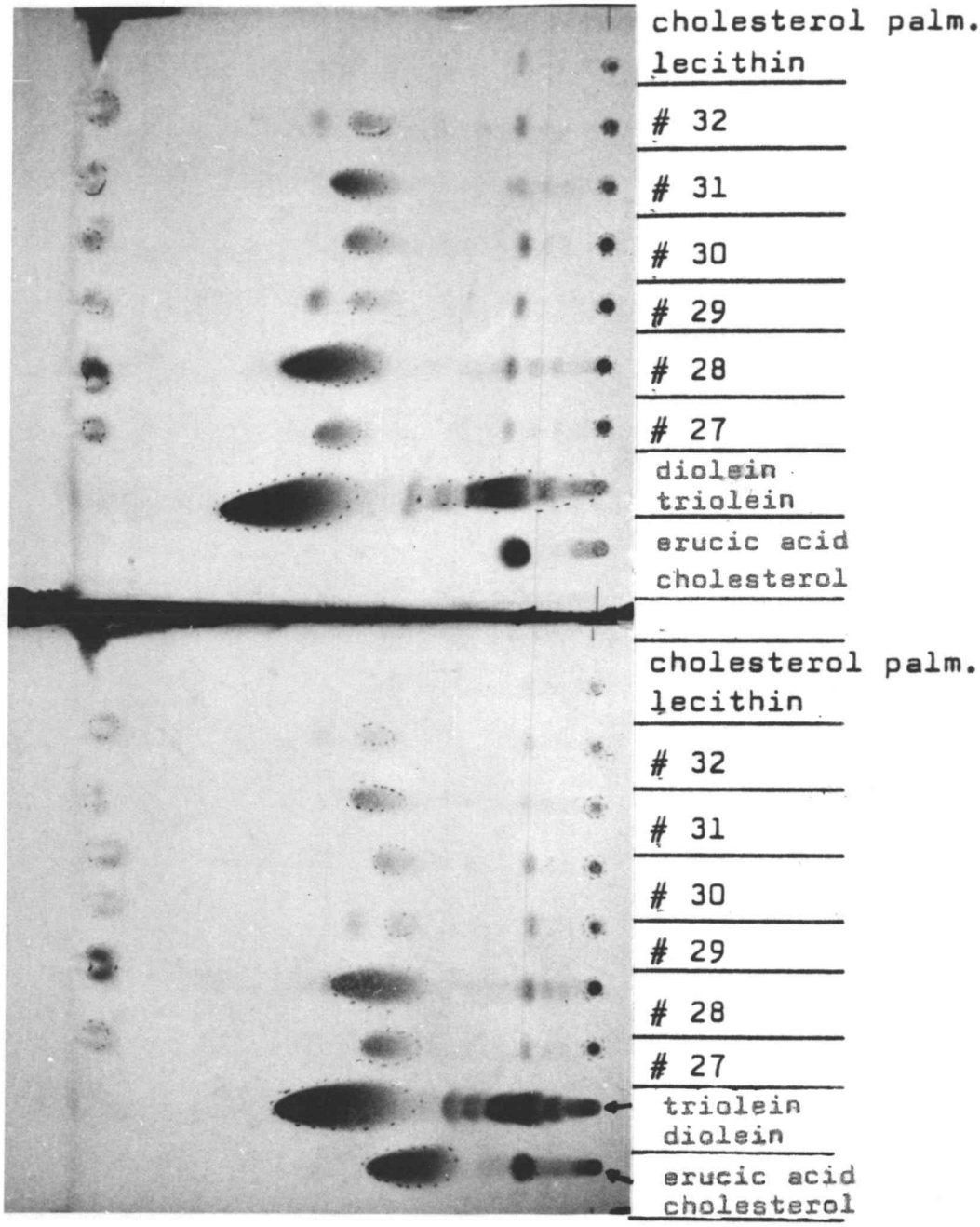

cholesterol palm.
lecithin
32
31
30
29
28
27
diolein
triolein
erucic acid
cholesterol
cholesterol palm.
lecithin
32
31
30
29
28
27
triolein
diolein
erucic acid
cholesterol

Plate IV

A low power micrograph of very electron dense hepatic tissue of the male frog showing the relationship between circulatory components and parenchymal cells. The capillary at the bottom is surrounded by an endothelial cell (En) which in turn is separated from the parenchymal cell by a space of Disse (SD). The surface of the hepatic cell is folded into many microvilli (Mv). Some inclusions within the parenchymal cell are a tubular endoplasmic reticulum (ER_t), lipid droplets (L) and a nucleus containing a nucleolus (Nu). Osmium tetroxide--Uranyl acetate stain and mounted on bare grids (6, 300X)

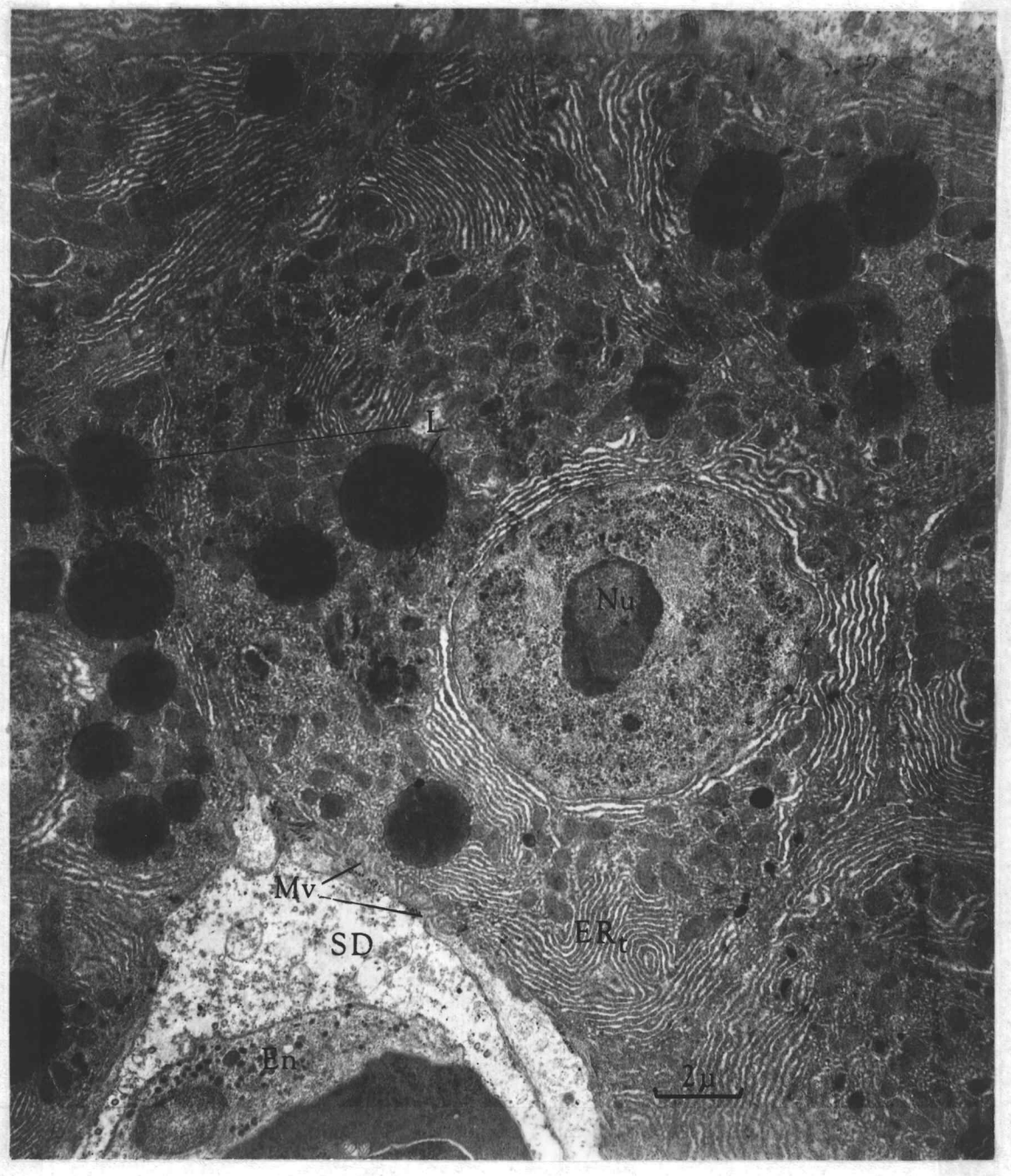

L
Nu
Mv
SD
ERt
Bm
2μ

Plate V

A hepatic parenchymal cell from a male frog containing a nucleolus (Nu) within a nucleus, lipid droplets (L), tubular endoplasmic reticulum (ER) and dense bodies (DB). Osmium tetroxide--Uranyl acetate and mounted on bare grids (9,400 X)

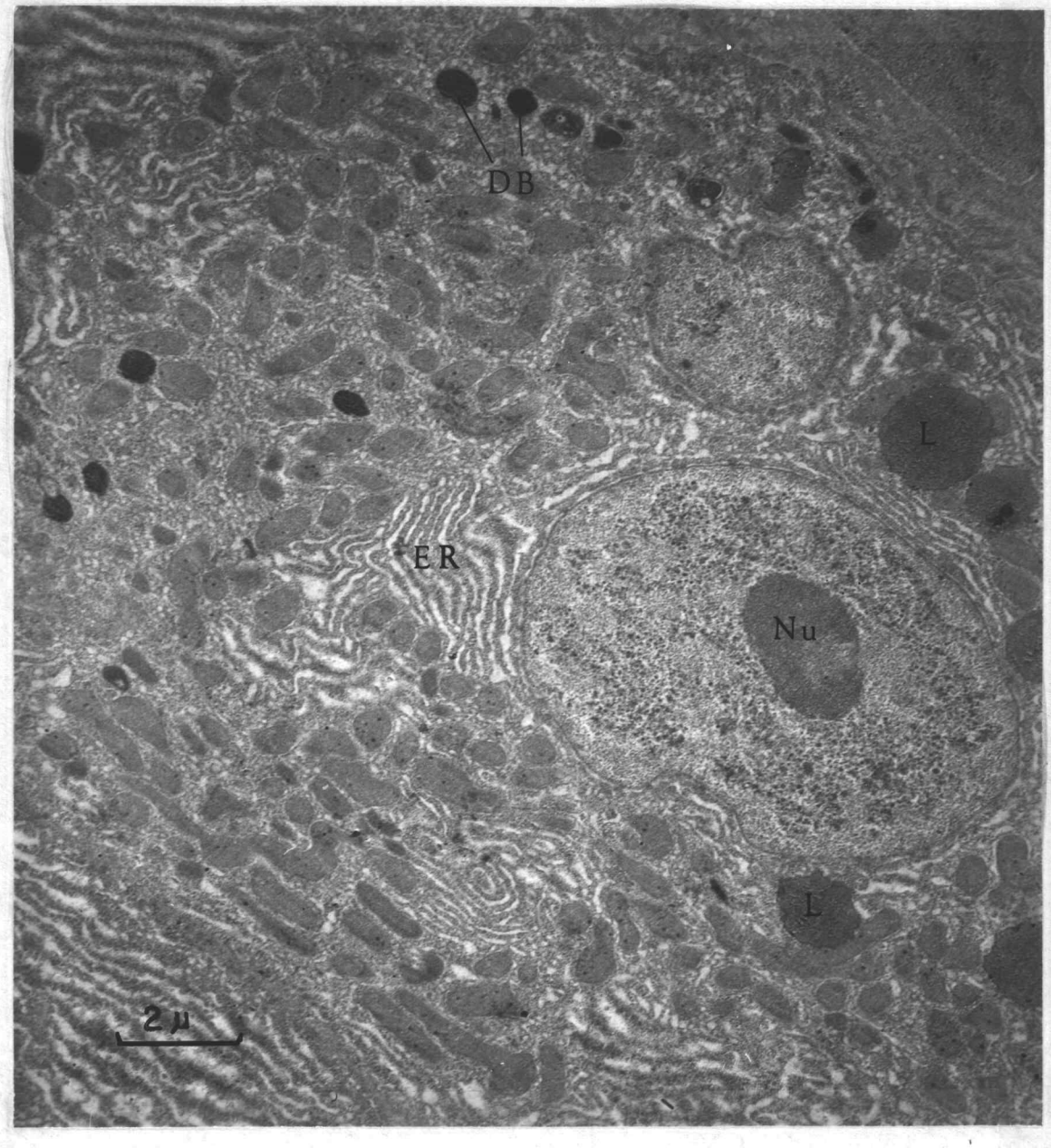

DB
L
ER
Nu
L
2μ

Plate VI

The lipid droplets (L), glycogen (G) and small dense mitochondria (M) are often clustered together between highly developed tubular endoplasmic reticulum (ER). Each bile canaliculus (BC) is lined with numerous finger-like microvilli and is formed by the convergence of four or five parenchymal cells. Osmium tetroxide--Uranyl acetate stain and mounted on bare grids (6, 500X)

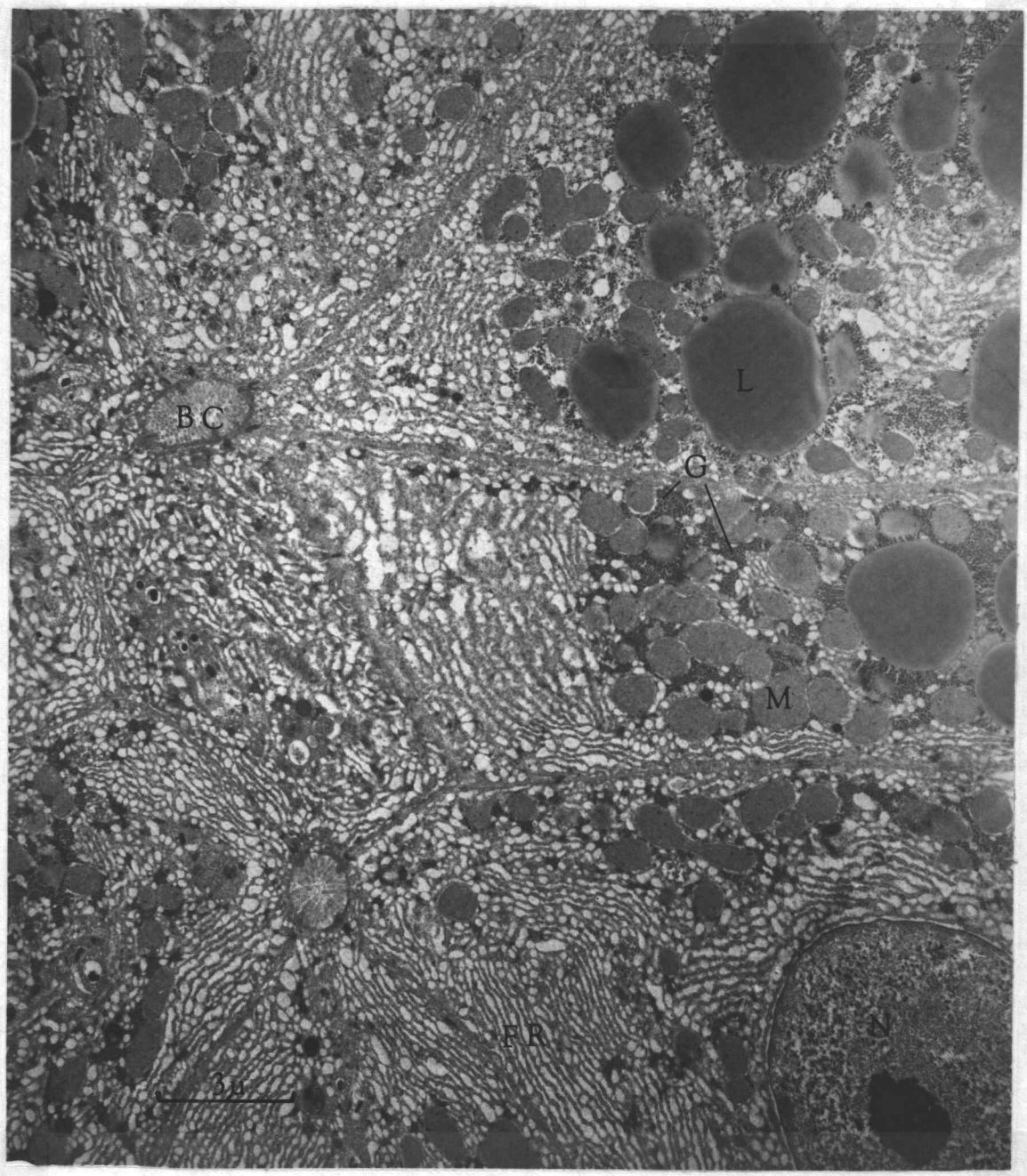

BC
L
G
M
FR
N
3μ

Plate VII

The close association between mitochondria (M) and lipid droplets (L) may be seen in this enlarged electron micrograph. Note that glycogen (G) is also found dispersed throughout the rough vesicular endoplasmic reticulum (ER_v) of the three cells. Osmium tetroxide--Uranyl acetate counterstained with lead citrate (13, 900X)

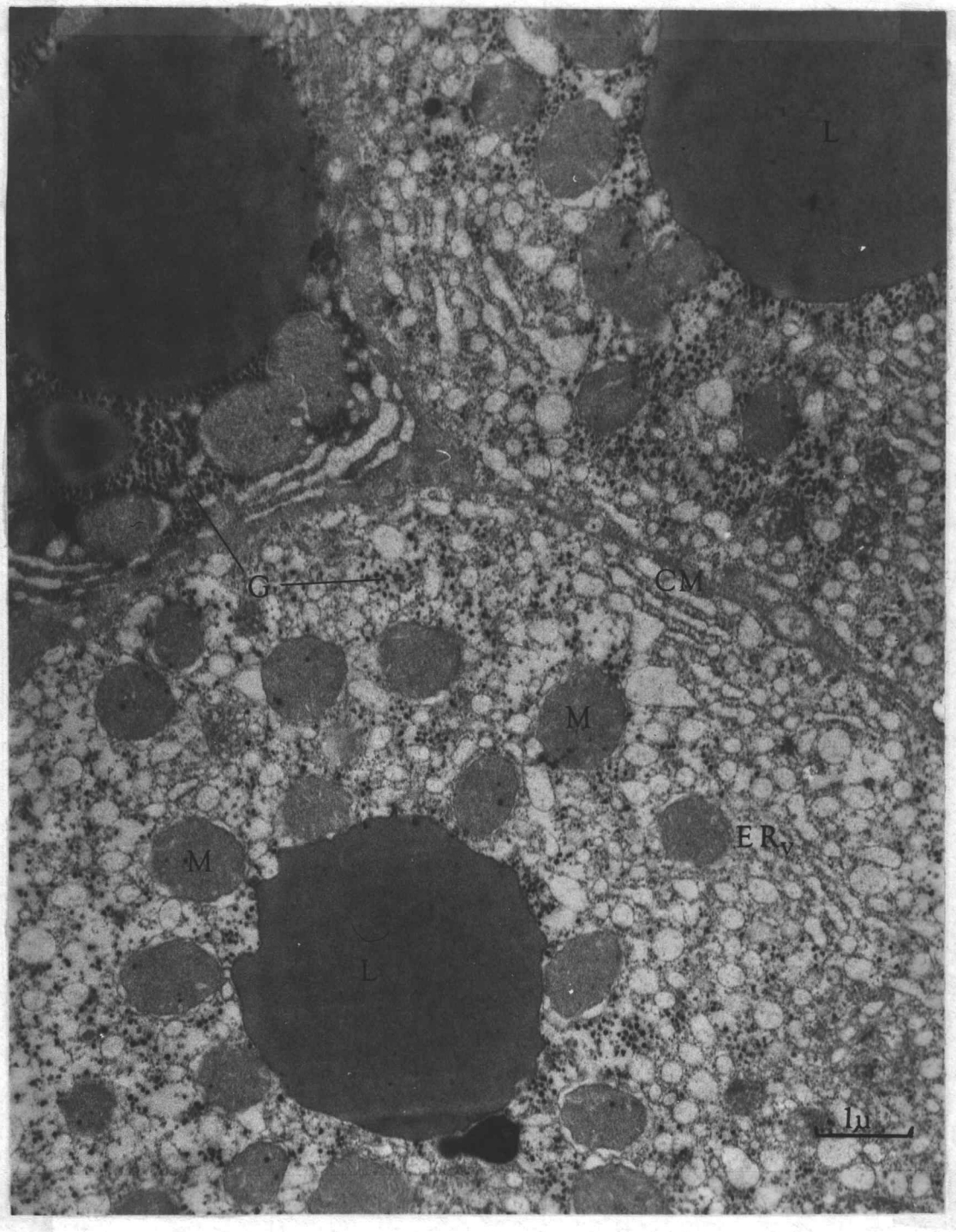

L
G
CM
M
M
ERv
L
1μ

Plate VIII

A contrast between parenchymal cells having a vesicular endoplasmic reticulum (ER_v) and a tubular endoplasmic reticulum (ER_t) characteristic of the more electron dense cells. Note that the mitochondria (M) do not possess clear cristae but are small dense homogeneous bodies interspersed between the lipid droplets (Li). Osmium tetroxide--uranyl acetate stain and mounted on bare grids (7, 100 X)

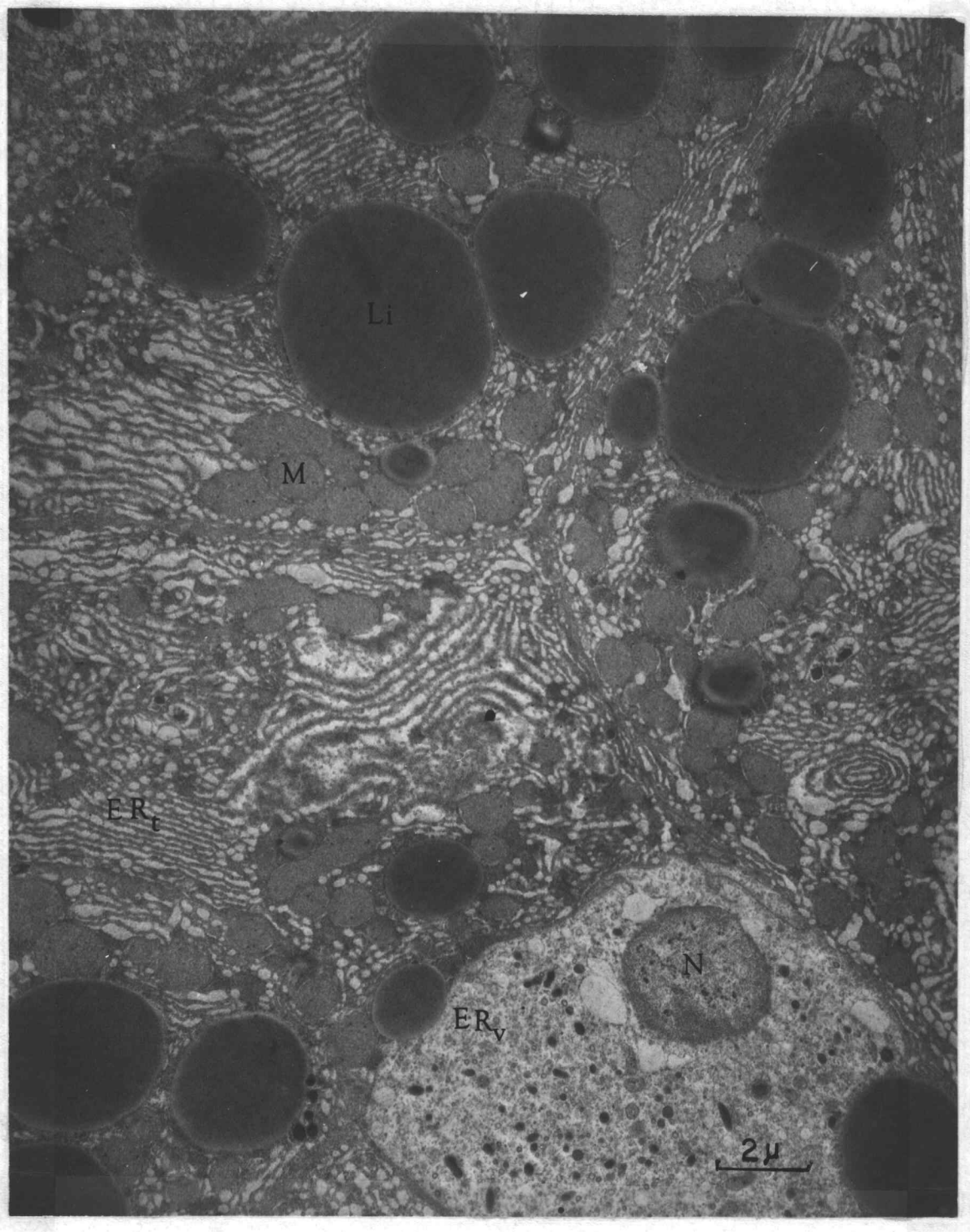

Li
M
ER_t
N
ER_v
2μ

Plate IX

Hepatic cell subjected to a 20 minute treatment in cold acetone containing magnesium chloride for the removal of neutral lipids. Note vesicles (V) remain to mark the location of previous lipid deposits. Some lipid material appears to remain in the vesicles. Shrinkage of the tissue has caused clumping of the chromatin in the nucleus (N). Note some dense bodies (DB) were not extracted with the lipid. Osmium tetroxide--Uranyl acetate stain and mounted on bare grids (10, 000 X)

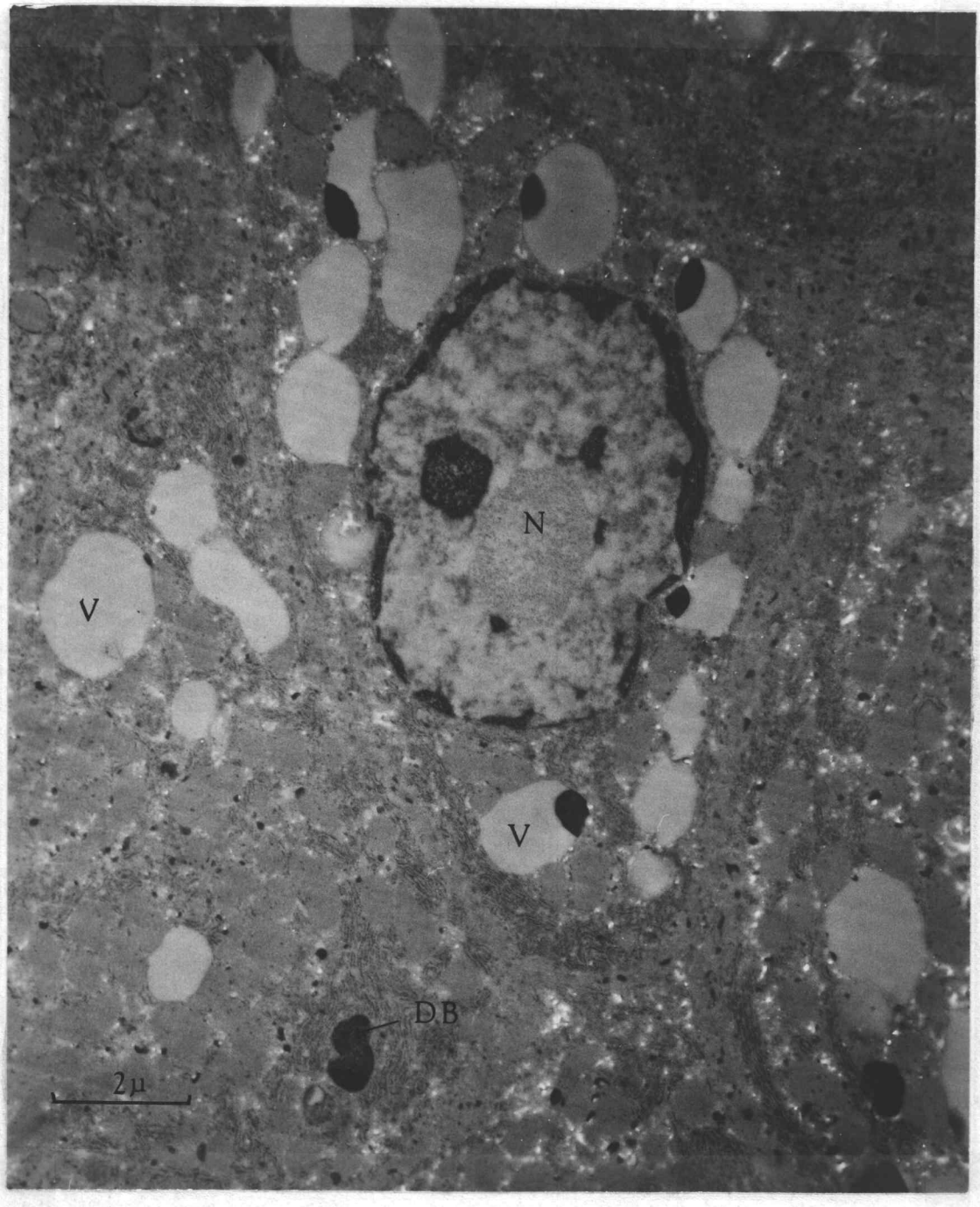

N
V
V
D.B
2μ

Plate X

Vesicles partially filled with lipid (L) resulting from mobilization occurring just prior to ovulation. As the lipids are removed the interface (I) around each lipid droplet shrinks leaving a sparse vesicular endoplasmic reticulum. Note the dense bodies (D) within the well oriented tubular endoplasmic reticulum and near the bile canaliculus. Osmium tetroxide--Uranyl acetate counter-stained with lead citrate (5, 200 X)

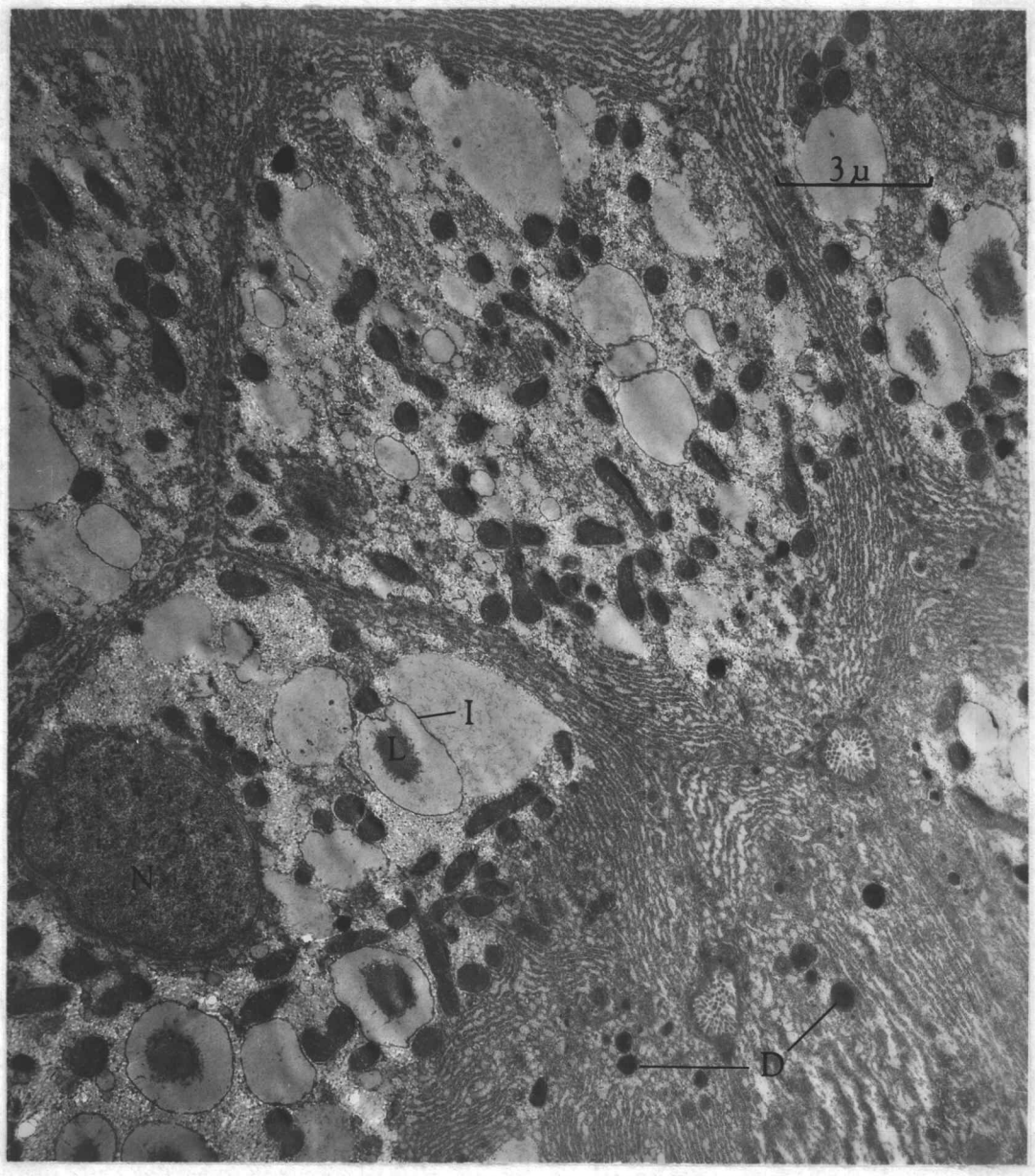

3 μ
I
L
N
D

Plate XI

The relationship between the shrinking lipid droplet (L) and the surrounding interface (arrow) may be seen in this high power electron micrograph. Again the mitochondria (M) are found in close proximity to the lipid droplets. Dense bodies (DB) are found dispersed throughout the endoplasmic reticulum. Osmium tetroxide--Uranyl acetate counterstained with lead citrate (16, 700 X)

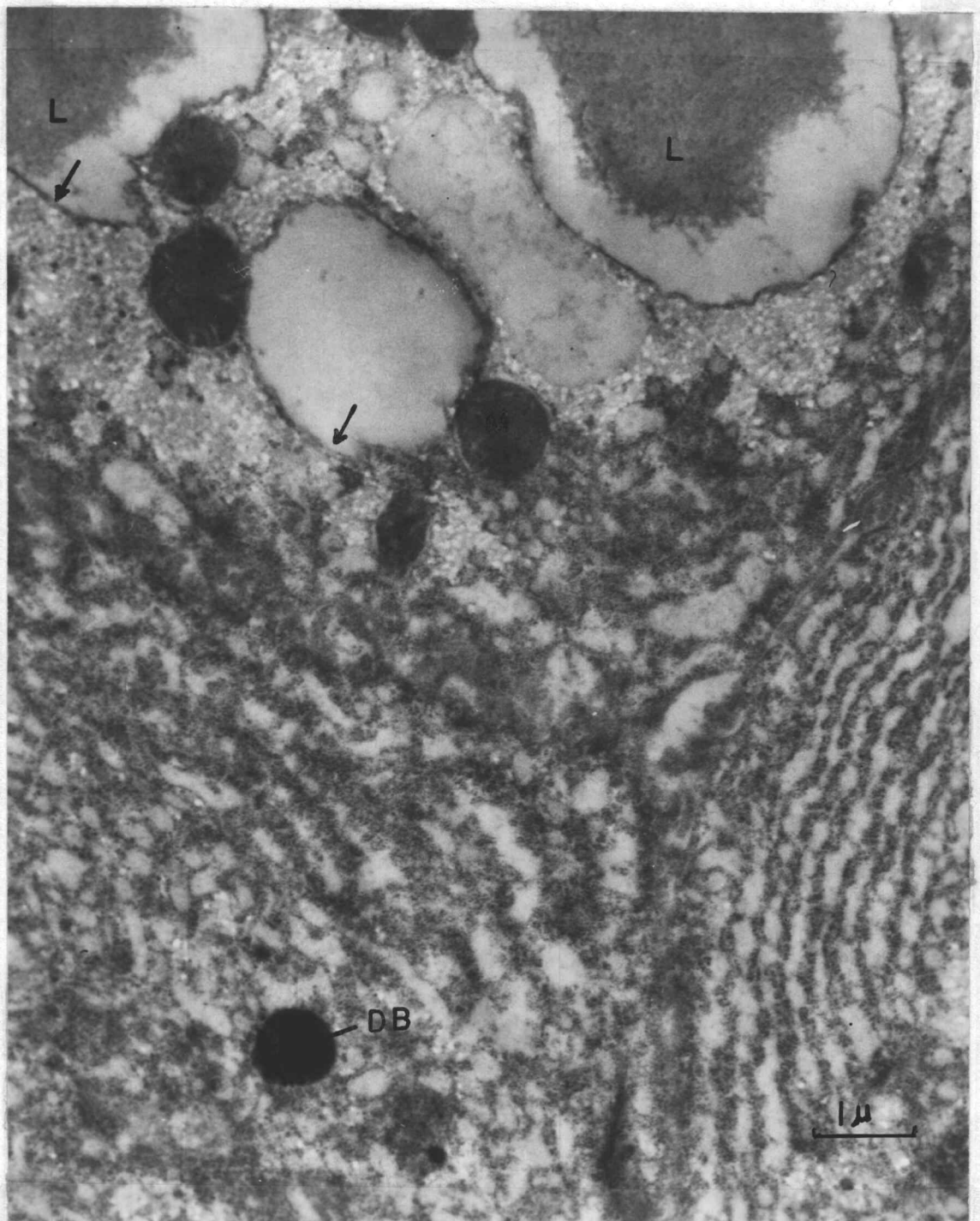

L
L
DB
1μ

Plate XII

A low power micrograph of the female hepatic cells during ovulatory period showing an endoplasmic reticulum (ER_v) filling in the space left by the diminishing lipid (L). Melanocytes containing numerous melanin granules (MG) were frequently found in the tissue sections. The square encloses the area shown on Plate XIII. Osmium tetroxide--Uranyl acetate counterstained with lead citrate (4, 300 X)


MG
L
N
ER
4μ

Plate XIII

The vesicular endoplasmic reticulum (ER_v) is less dense around the remaining lipid droplets (L) suggesting that it has not completely filled in the space after the lipid mobilization. Numerous mitochondria (M) are found in close proximity to the irregularly shaped lipid droplets (L). Osmium tetroxide--uranyl acetate counterstained with lead citrate (12, 100 X)

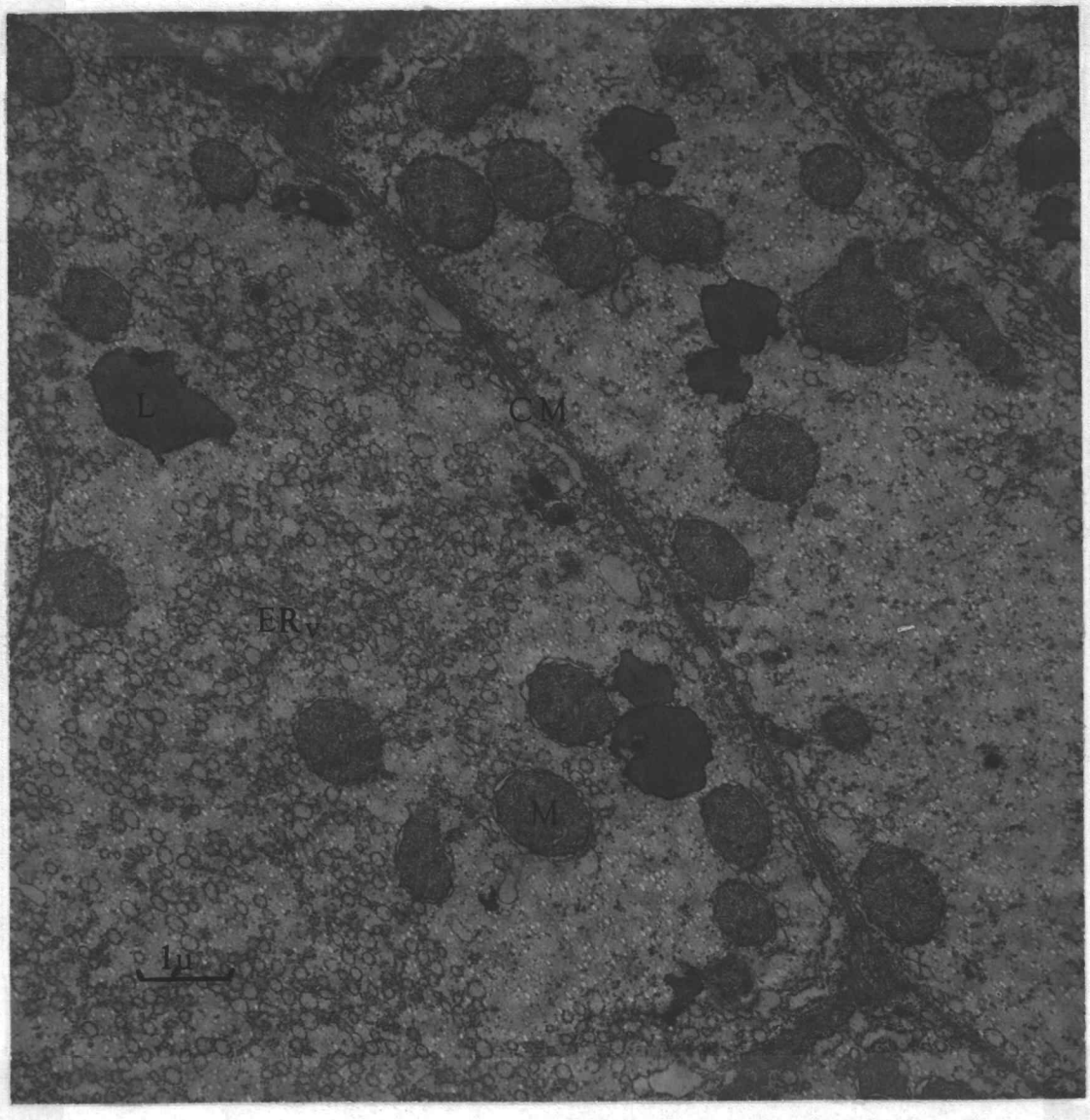

L
CM
ERv
M
1μ

Plate XIV

The reorientation of morphological structures has been brought about after the removal of lipid. Note the expanded areas of vesicular endoplasmic reticulum (ER_v) and the remains of a few irregularly shaped lipid droplets (L). The morphology of the tubular endoplasmic reticulum (ER_t), mitochondria (M) and nucleus (N) appear to be unchanged. Osmium tetroxide--Uranyl acetate counterstained with lead citrate (5,100X)

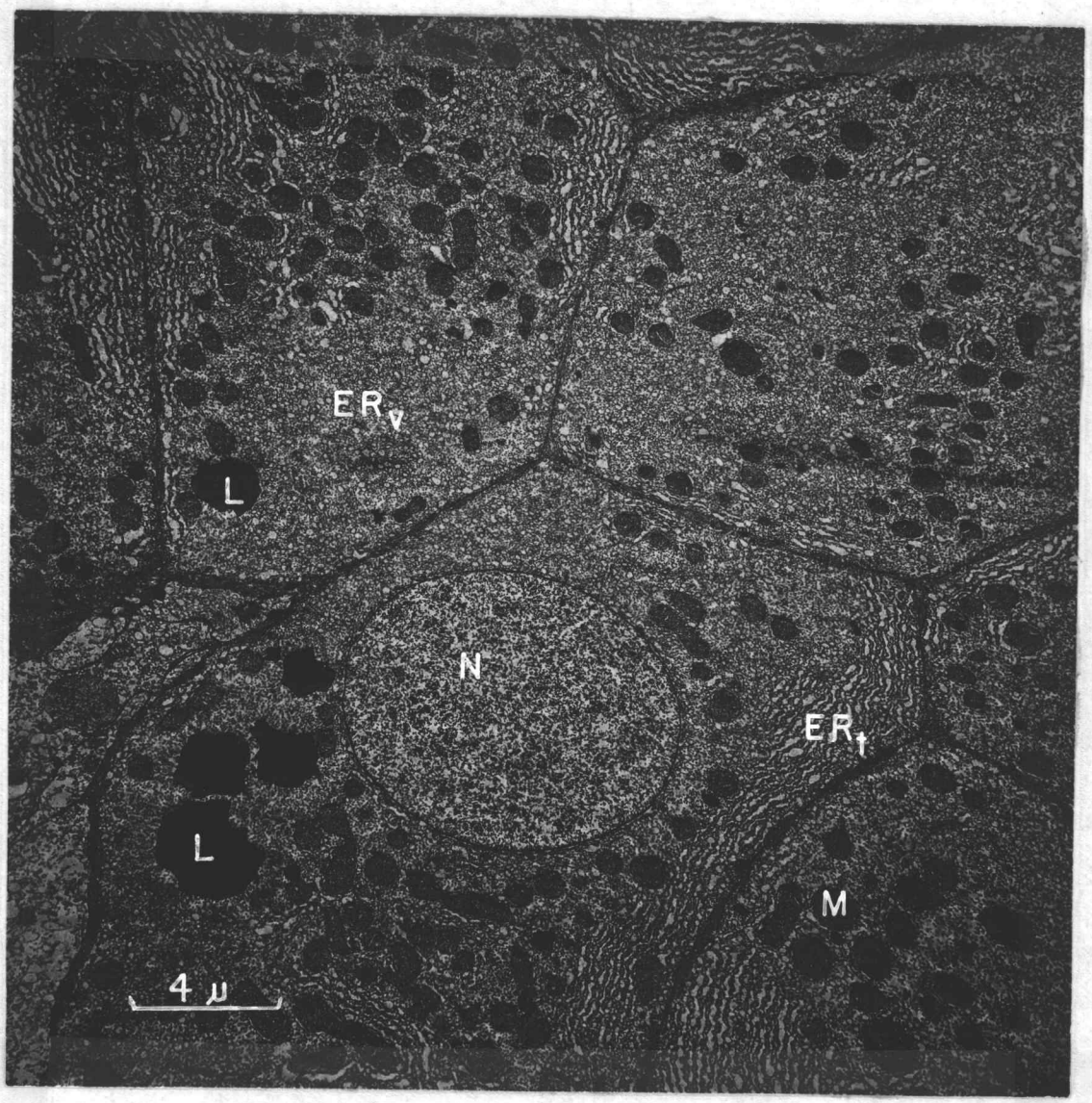
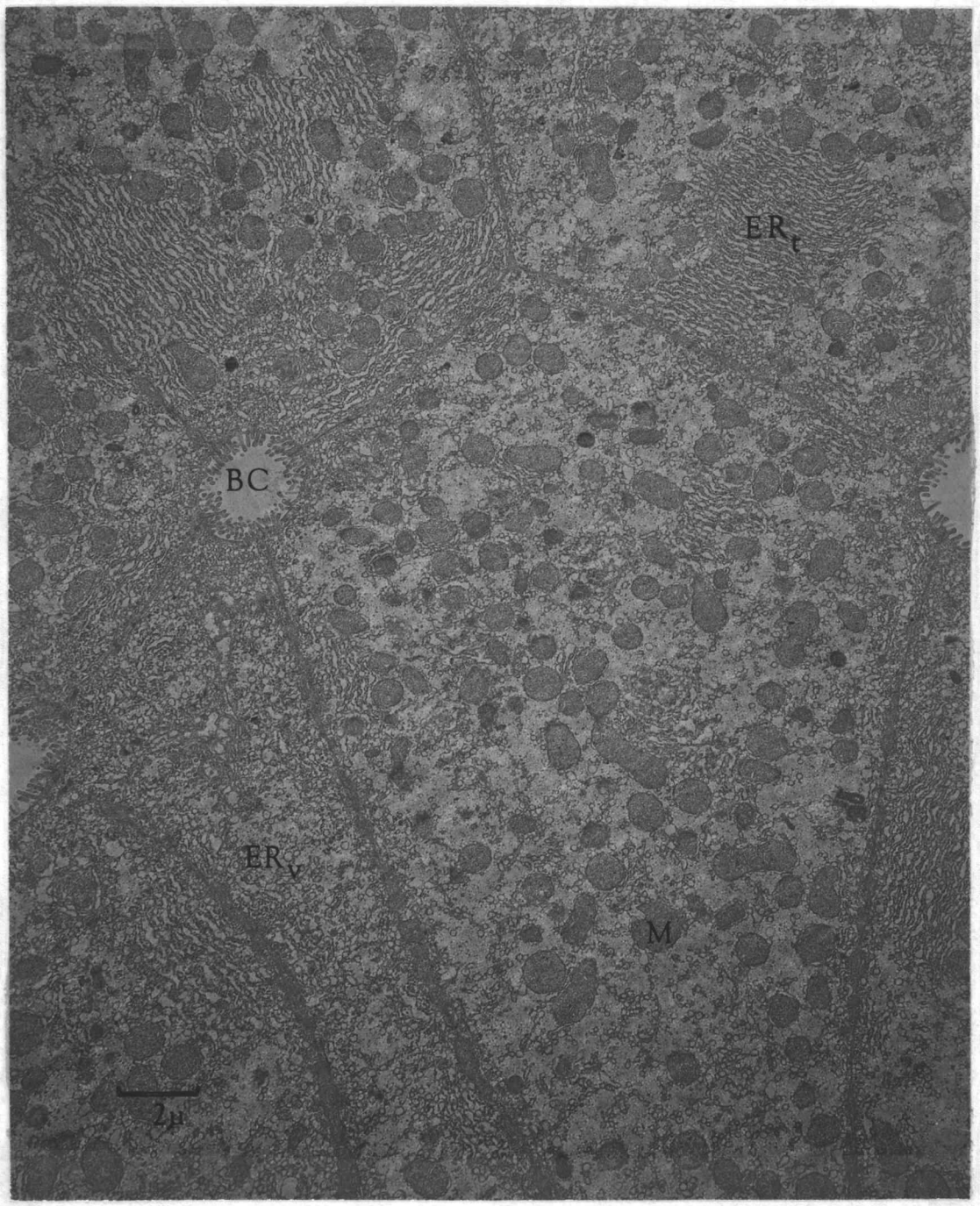

ER$_v$
L
N
ER$_f$
L
M
4 μ

Plate XV

At the conclusion of the reproductive cycle very few lipid droplets remain in the cytoplasm of the female hepatic cells. The mitochondria (M) are dispersed throughout a vesicular endoplasmic reticulum (ER_v). Irregular clumps of well developed tubular endoplasmic reticulum (ER_t) may be found. Note the bile canaliculus (BC) at the convergence of four cells is lined with microvilli. Osmium tetroxide--Uranyl acetate counterstained with lead citrate (6, 200 X)


ER$_t$
BC
ER$_v$
M
2μ