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Morphological and Biochemical Studies on Diethylnitrosamine-induced Hepatocarcinogenesis in Rat

Yutaka YOSHIDA, Noriyasu CHISAKA and Aiko KANEKO
Department of Pathology (Section 2), Sapporo Medical College
(Chief: Prof. T. Onoé)

Introduction

Magee[1,2] first reported that one of the carcinogenic nitroso compounds, dimethylnitrosamine (DMN) produced liver tumors. Schmähl[3] examined the carcinogenicity of diethylnitrosamine (DEN) using various animal species and showed that DEN was more carcinogenic and less toxic than DMN on rat liver.

Morphological, biochemical and histochemical studies on 3′-methyl-4-dimethylaminoazobenzene (3′-Me-DAB) carcinogenesis in rat liver have been performed in our laboratory[4,5,6,7,8]. It has been delineated that there is a marked fluctuation of various cell populations in the early stage of 3′-Me-DAB hepatocarcinogenesis, accompanied by the early appearance of α-fetoprotein (AFP) in the sera and the deviation to immature types of isozyme patterns of acid phosphatase, aldolase and non-specific esterase of hepatic tissue[7,8].

In the present study, histological, ultrastructural and biochemical investigations on the sequential process of DEN hepatocarcinogenesis were attempted, especially paying attention to the following problems: 1) Does there exist any precancerous change as seen in 3′-Me-DAB carcinogenesis? 2) Does there exist the fluctuation of various cell populations in the early stage of DEN carcinogenesis such as marked cholangiolar cell (so-called oval cell) proliferation which was observed in 3′-Me-DAB carcinogenesis[4,5]?

Materials and Methods

Male adult rats of Wistar strain, initially weighing 170–200 g, were individually caged at 20°C in an air-conditioned room. DEN was given orally in the drinking water in a daily dose of 3 mg/kg body weight[3]. Animals were fed on the Oriental solid diet (Oriental Yeast Co., Tokyo). The times when the animals were sacrificed were as follows: 2, 4 days, 1–6 week and thereafter every 2 weeks until 32 weeks after the commencement of DEN administration. Three rats were sacrificed at each time point. Thin slices were taken from each of the 5 lobes of the liver.

Light microscopy

Tissues were fixed in cold Carnoy's and/or 10% cold neutral formol solution for a few hours and also in 90% ethanol solution overnight. Paraffin sections were stained with Hematoxylin-Eosin (H–E), Toluidin Blue or periodic acid-Schiff (PAS). Epon-embedded semithin sections were stained with PAS-Toluidin Blue by the method of Cardono and Steiner[9].

Electron microscopy

Specimens of liver tissues were fixed for 30 min in 2% glutaraldehyde solution buffered at pH 7.4, post-osmificated, dehydrated in a graded series of ethanols, and embedded in Epon 812. Ultrathin sections were cut on a Porter-Blum MT-2 ultramicrotome, stained with lead citrate and/or uranyl acetate, and examined with a Hitachi HS-7D electron microscope.

Enzyme assay

After removal of liver slices for morphological examination, the remainder of each liver was placed in an ice-cold 0.25 M sucrose solution, and homogenized with a glass-Teflon homogenizer to make a 20% (W/V) homogenate in 0.25 M sucrose solution. The activity of glucose-6-phosphatase (G-6-Pase) was measured as described by Swanson[10], except for the use of 0.02 M Tris-maleate buffer (pH 6.75). The reaction mixture containing 0.25 M glucose-6-phosphate (Sigma Chem. Co., St. Louis) and 0.005 M $MgCl_2$ was incubated at 37°C for 10 min. Released inorganic phosphorus (Pi) was measured by the method of Fiske and Subbarow[11]. The esterase activity was determined with β-naphthyl acetate as a substrate, by a slightly modified method of Nachlas and Seligman[12]. The amount of β-naphthol released was determined directly at 328 nm at room temperature (approx. 24°C) at intervals of 30 sec for 10 min.

Isozyme pattern of esterase

Electrophoresis on the cellulose acetate membrane (Sepraphore III, Gelman, 2.5× 17 cm) was carried out using veronal buffer (pH 8.6, I=0.07) at 250 V for 2 hr at 10°C. After electrophoresis, the strips were stained on filtered Noble agar (Difco) solution (1%) containing 0.025 M veronal buffer (pH 7.2), 0.4 mg/mℓ β-naphthyl acetate dissolved in 0.04 mℓ of acetone and 0.5 mg/mℓ Fast Blue RR. Incubation was made for about 10 min at 37°C.

Assay of serum AFP

AFP in the serum was tested by double diffusion in agar gel with absorbed anti-AFP antiserum. Micro-Ouchterlony plates were made with special Noble agar (Difco), 1% in saline solution. Dr. Watabe* kindly performed a radioimmunoassay of serum AFP for each sacrificed animal.

Estimation of nuclear volume

The light microscopic specimens were photographed so as to exclude non-hepatocytes such as cholangiolar cells and endothelial cells. The size of the photographs was 20.3×25.4 cm (magnified 100 times). Nuclear diameters were measured with a Particle Size Analyzer TGZ 3 (Carl Zeiss). The cells measured were only hepatocytes with almost fully round nuclei. Transition from diameter distribution to volume distribution is: $dV=d\left(\frac{D^3}{6}\pi\right)=\frac{\pi}{2}D^2dD$ D: diameter. V: volume. The abscissa values were multiplied by $\frac{\pi}{6}D^2$.

* Department of Biochemistry, Hokkaido University School of Medicine, Kita-15-jo, Nishi-7-chome, Kita-ku, Sapporo 060.

Results

Light microscopic observations

Two or three days after administration of DEN, hepatocytes in the periphery of hepatic lobules were slightly degenerated and showed an increase in cytoplasmic eosinophility. After 2 weeks there were moderate to marked number of mitotic figures in the peripheral or midzonal areas of the liver lobules (Photo 1). Centrilobular regions showed no degenerative change. In the midzonal areas of hepatic lobules were observed small cell populations whose perinuclear cytoplasm was stained lightly due to the elution of the cytoplasmic material during the preparation for histological examination (Photo 2). These cells were called hydropic cells tentatively. The hydropic cells were revealed to store abundant glycogen by the epon-embedded semithin section stained with the method of Cardono and Steiner[9] (Photo 3). After 3 weeks of DEN administration, small basophilic cells were observed around the portal areas and adjacent to those cells, the hydropic cells were frequently observed. These basophilic cells slightly increased after 10 weeks of DEN administration and appeared as small foci from 12 weeks on, which seem to be coincident with the hyperbasophilic foci described by Daoust et al.[19,20] (Photo 4, 5) After 14 weeks, degenerative changes in the hepatocytes reappeared, although to a small extent, and even necrotic changes were observed. Marked mitotic figures were again observed after 16 weeks of DEN administration (Photo 6, 7). By the 18th week the hyperbasophilic foci seemed to develop into hyperplastic nodules through pronounced mitosis in the preceding stage (Photo 8). Liver tumors were regularly observed at 24 weeks of administration and thereafter. The histology of almost all the tumors revealed well-differentiated hepatocellular carcinoma (Photo 9). Metastases to the lungs were observed in two rats (Photo 10). Throughout the carcinogenic process the cholangiolar cell (so-called oval cell) proliferation was not observed.

Electron microscopic observations

Ultrastructurally, the majority of basophilic cells mentioned above had irregular shaped nuclei of small size. The cytoplasmic matrix was filled with free ribosomes and polysomes in accordance with their cytoplasmic basophility in the light microscopic specimens stained with Toluidin Blue. Round or ovoid mitochondria were evenly distributed in the cytoplasm. Some of mitochondrial cristae were meandering characteristically. The Golgi apparatus was well developed and located in the perinuclear region. The cytoplasm was almost free from glycogen particles. Microbodies and lysosomes were also noticed (Photo 11, 12, 13).

Enzyme assay

Fig. 1 shows changes in the total activities of G-6-Pase and esterase throughout the experimental course. The activities of these enzymes decreased gradually up to 4 days after initiation of DEN administration. At the end of the 2nd and 16th week of DEN administration, when marked mitosis was observed, both enzyme activities showed a characteristic pattern: i, e., the activity of G-6-Pase slightly decreased, while the esterase activity increased. Thereafter in the stage of nodule formation at the 18th

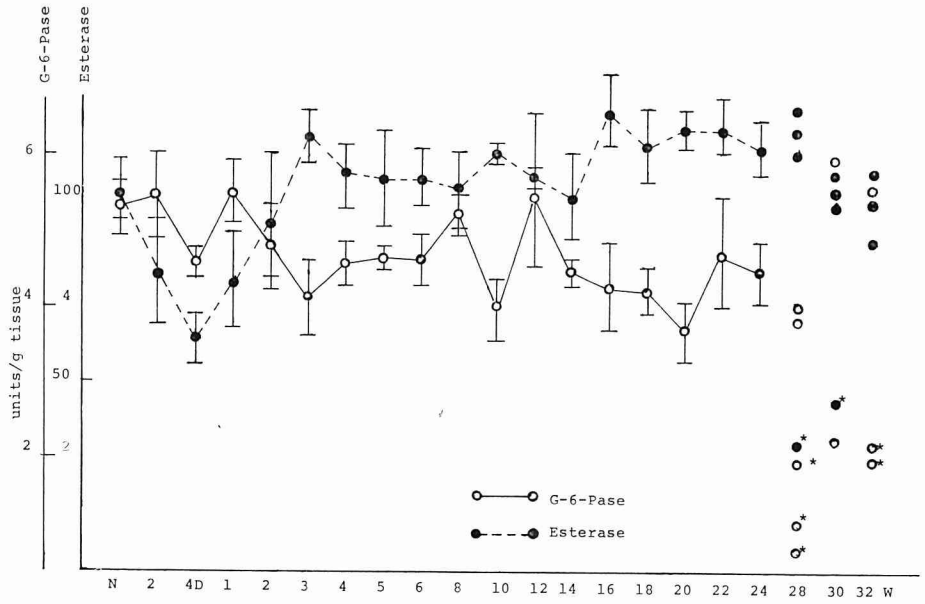

Fig. 1 Changes of enzyme activities during DEN carcinogenesis.
* carcinoma tissue. N: normal adult rat liver. D: day W: week.
Unit: G-6-Pase, μmole Pi/min; esterase, μmole β-naphthol/min.

week of DEN administration, the activity of G-6-Pase showed a gradual decrease until the primary carcinoma arose. The esterase activity increased contrarily till the formation of cancer.

Isozyme pattern of esterase

At the end of the 3rd week when abundant mitotic figures were first observed, the L-I of esterase, moving farthest towards the anode, appeared more strongly than in normal. The intensity of L-I increased gradually thereafter. At the same time, L-V, the far cathodic band, appeared faintly and almost disappeared by the stage of the appearance of hyperplastic nodules, and again was observed at the 18th week of administration. L-V continued to appear till the carcinoma arose (Fig. 2).

Estimation of nuclear volume

As shown in Fig. 3, the incidence curve of the nuclear volume of the hepatocytes manifested itself in three peaks in the control rats as reported by Grundman and Sieburg[13]. It has been known that the main class of the hepatocytes in adult rat liver is the tetraploidy. Therefore it is most likely that the middle peak represents the tetraploidy, and the left peak and the right peak, the cell class of the diploidy and the octaploidy, respectively. During 4 to 10 weeks of DEN administration, the curve was displaced towards the right. At the 18th week of administration, however, small-sized diploid hepatocytes increased strikingly and so did the polyploidy.

Assay of AFP in the serum

The concentration of AFP in the serum during DEN carcinogenesis is shown in Fig. 4. After the formation of hyperplastic nodules at the 18th week of DEN adminis-

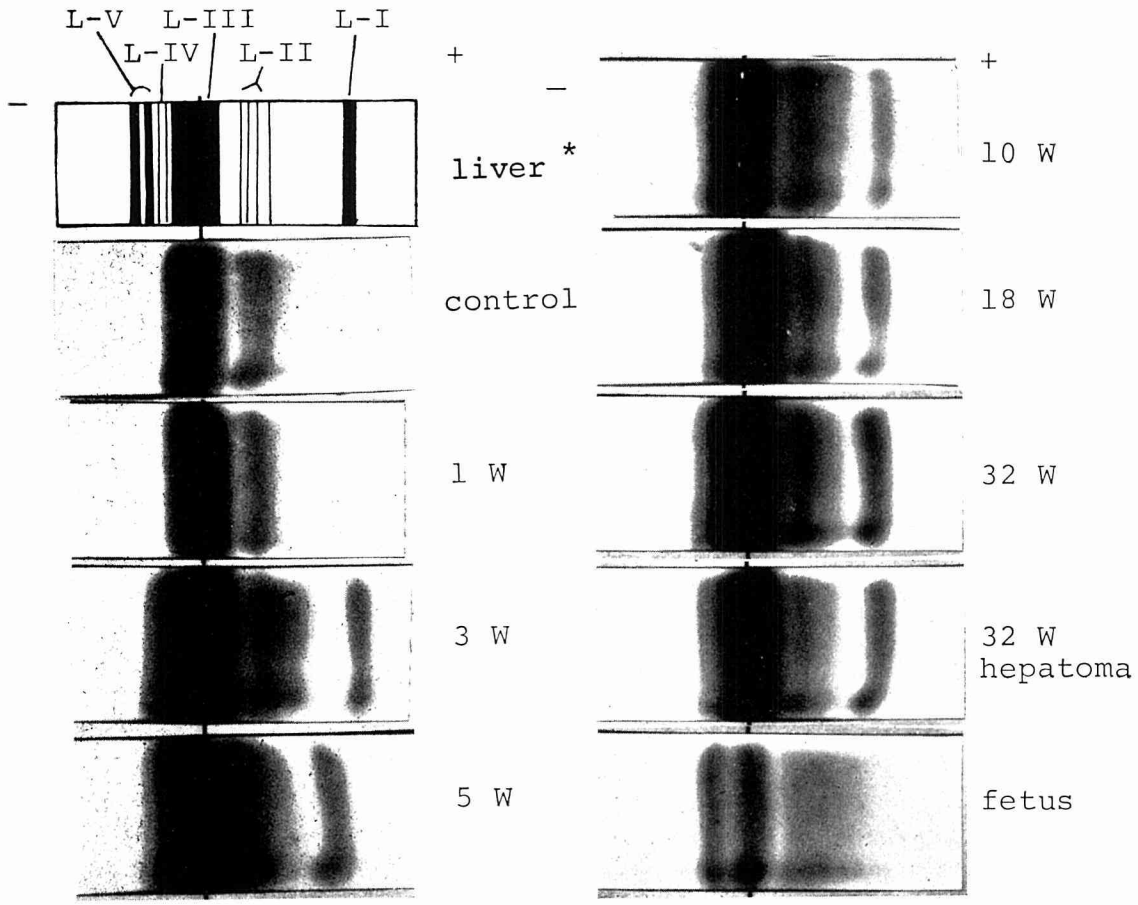

Fig. 2 Esterase pattern during DEN carcinogenesis.
* Illustrative tracing of electropholetic pattern.

tration, AFP appeared slightly in the serum. The maximum AFP concentration was coincident with the induction of cancer. However, in the early stage of carcinogenesis, a significant increase of AFP was not conspicuous.

Discussion

The first histological change within 2 weeks of DEN administration was a slight eosinophilic degeneration of hepatocytes in the periportal area. It was followed by a marked proliferation of hepatocytes, the peak of which was observed at the end of the 2nd week. This stage of degeneration and proliferation seemed to reflect an acute toxic effect of DEN upon the hepatocytes. As shown in Fig. 1, degeneration of hepatocytes was accompanied by decreases in G-6-Pase and esterase activities. The appearance of small basophilic cells was observed after these mitoses of hepatocytes in this stage. The electron microscopic findings regarding these cells were essentially similar to those of regenerating hepatocytes at the 24-hr period after partial hepa-

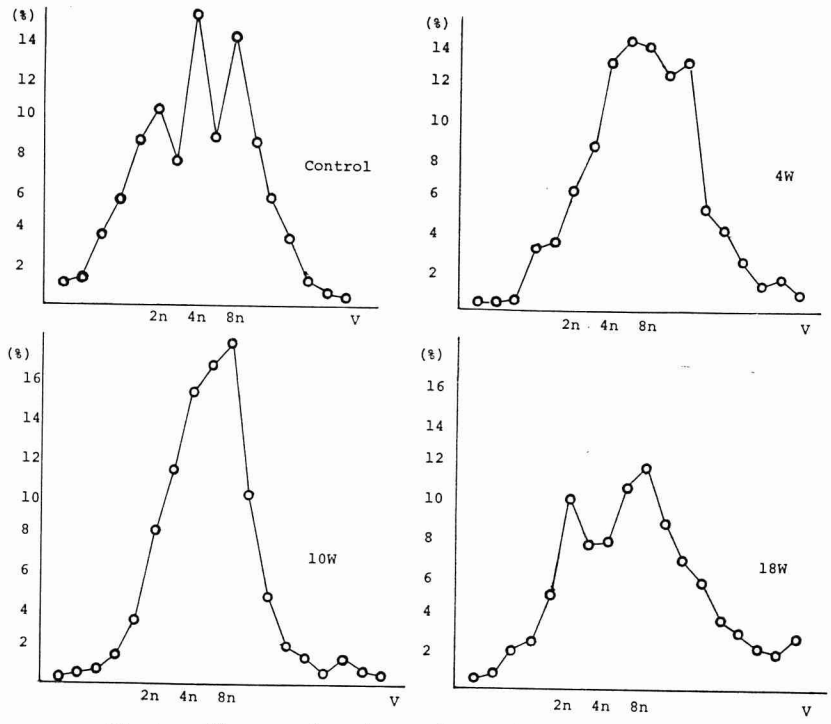

Fig. 3 Changes of nuclear volume of hepatocytes during DEN carcinogenesis.

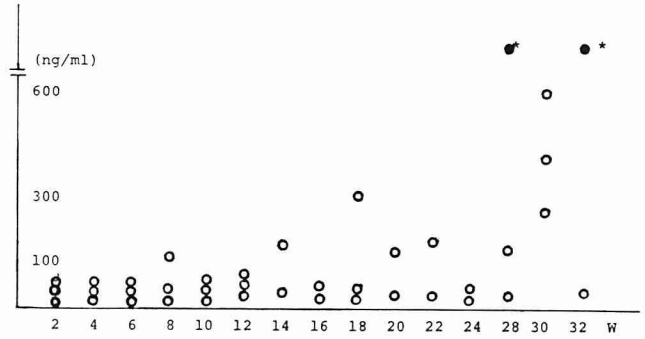

Fig. 4 AFP concentration during DEN carcinogenesis.
* positive in micro-Ouchterlony method.

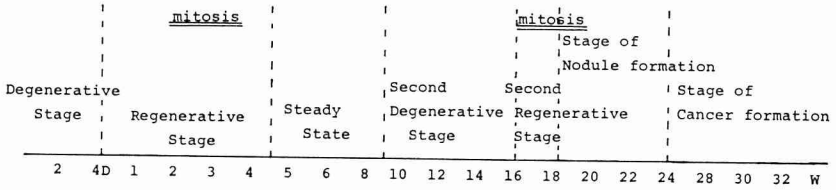

Fig. 5 Carcinogenic stages during DEN carcinogenesis.

tectomy as reported by Kimura[14]. After 2 weeks of DEN administration, G-6-Pase activity gradually decreased in spite of a slight increase of esterase activity as shown in Fig. 1. This pattern of enzyme activities is also similar to that of regenerating rat liver after partial hepatectomy.

Changes of the esterase isozyme pattern were observed in parallel with the histological changes. Kaneko et al.[7,15] described the heterogeneity of esterase of hepatic tissue in normal condition and the carcinogenic process by 3′-Me-DAB. The esterase isozyme pattern changed from the adult type to the infant type revealing a strong intensity in L–I of esterase at the 3rd week of DEN administration as shown in Fig. 2. This pattern also resembled that of regenerating liver. It seemed that the increase in the intensity of L–I reflected the regenerating process in which the small basophilic cells might play a crucial role.

L–V, a more immature type of esterase, appeared temporarily at the 3rd week following increase in the number of mitotic figures. The appearance of esterase isozyme L–V has been observed also in the early stage of 3′-Me-DAB carcinogenesis[7].

No evident increase of serum AFP could be observed in the early stage of DEN carcinogenesis. On the other hand, in the early stage of carcinogenesis by 3′-Me-DAB, the deviation of the isozyme pattern of esterase to an immature type was observed to be accompanied by a transient appearance of AFP in sera[21]. Dempo et al.[16] proved that almost all the AFP producing cells were transitional hepatocytes derived from proliferating oval cells at this stage. This difference between in the early stage of carcinogenesis by DEN and 3′-Me-DAB might be ascribed to the difference in character with reference to the degree of dedifferentiation between the oval cells or transitional hepatocytes and the basophilic cells both of which seem to play a leading role in regeneration of the hepatic tissue damaged by the carcinogen.

The hydropic cell population remained observable throughout the carcinogenic process. Bannasch[17,18] reported an appearance of hepatocytes characterized by enhanced glycogen storage in N-nitrosomorpholine-intoxicated rat liver and considered it as a precancerous reaction of cells. In the present study, however, the intimate correlation between basophilic cells and hydropic cells was observed only in respect to their location within the liver lobule, and the role of hydropic cells in the precancerous stage remained obscure.

After 12 weeks of DEN administration small groups composed of small basophilic cells were frequently observed. These groups of cells are considered to coincide with hyperbasophilic foci mentioned by Daoust et al.[19,20] At the 14th week of DEN administration and thereafter, a slight degeneration and necrosis of parenchymal cells again began to be observed, accompanied by a slight decrease in the activities of G-6-Pase and esterase. This stage was followed by the regenerating process where a considerable number of mitotic figures were recognized in the liver tissue. As shown in Fig. 1, the G-6-Pase and esterase activities showed a characteristic pattern in this stage as described above.

At this stage when the second mitotic peak of hepatocytes was again observed, L–I of esterase was more intense and L–V reappeared, and this pattern was maintained up

to the stage of formation of cancer as shown in Fig. 2. The total activity of esterase slightly increased after 16 weeks in consequence of the appearance of L-V and L-I with the maintenance of L-III.

As shown in Fig. 3, the nuclear volume of hepatocytes was shifted towards the right during 4-10 weeks of DEN administration. This suggests that the proportion of polyploid cells in hepatocytes increased in this period. At the 18th week, small diploid hepatocytes increased strikingly as shown in a peak at the left side. This increase may be considered to reflect the second regeneration of hepatocytes at the 16th week of DEN administration. After this stage hyperplastic nodules were formed. In this stage of nodule formation, L-I and L-V of esterase were again detected. Carcinoma began to be induced around the 24th week of DEN administration. Therefore it is considered in DEN carcinogenesis that at least the existence of two proliferative stage of hepatocytes between degeneration and regeneration are essential to produce cancer. These sequential carcinogenic stages are summarized in Fig. 5.

Summary

The precancerous changes of rat liver induced by DEN were studied by sequential morphological and biochemical investigations.

1. By light microscopic observations, the initial hepatic change was a slight eosinophilic degeneration of periportal hepatocytes and was followed by a regenerating process in which marked mitotic figures of hepatocytes were observed after 2 weeks of DEN administration, resulting in appearance of small basophilic cells. Ultrastructurally, these samll basophilic cells were similar to those of the regenerating hepatocytes after partial hepatectomy. Biochemically, at the end of the 3rd week of DEN administration, the esterase isozyme patterns deviated to an immature liver cell type and the regenerating basophilic cells seem to be responsible for this phenomenon.

2. Marked mitotic figures were again observed after 16 weeks of DEN administration. After these mitoses, the hyperplastic nodules were formed. Some of these nodules seemed to develop into hepatoma. At this stage the esterase isozyme patterns deviated to an immature liver cell type the same as that seen in carcinoma tissue.

3. Therefore it is considered in DEN carcinogenesis that at least the existence of two proliferative stage of hepatocytes are essential to the induction of cancer. The small basophilic cells are considered to play a crucial role in the early stage of DEN carcinogenesis.

4. No remarkable cholangiolar cell (so-called oval cell) proliferation could be recognized throughout the entire course of DEN hepatocarcinogenesis. No evident increase in serum AFP could be detected in the early stage of DEN carcinogenesis.

Acknowledgement

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EXPLANATION OF PLATES

Photo. 1 The liver of a rat in the 2nd week of DEN administration. Moderate mitotic figures (indicated by arrow) are seen in the midzonal area of hepatic lobule. C: Central vein. P: Portal area. H-E. ×100.

Photo. 2 In the 6th week. This shows a hydropic cell population. H: Hydropic cell. H-E. ×100.

Photo. 3 In the 6th week. Semithin section stained by the method of Cardono and Steiner. Note that the hepatocytes in the midzonal area contain abundant glycogen within the cytoplasm (in the photograph, stained dark). ×500.

Photo. 4 In the 12th week. Small basophilic cells (B) increased and appeared as small foci. H-E. ×40.

Photo. 5 Higher magnification of Photo 4. Small basophilic foci among the hydropic cells are seen. H-E. ×100.

Photo. 6 In the 16th week. Marked mitotic figures (indicated by arrow) are again observed. H-E. ×100.

Photo. 7 Higher magnification of Photo 6. Marked mitotic figures are seen (arrow). H-E. ×250.

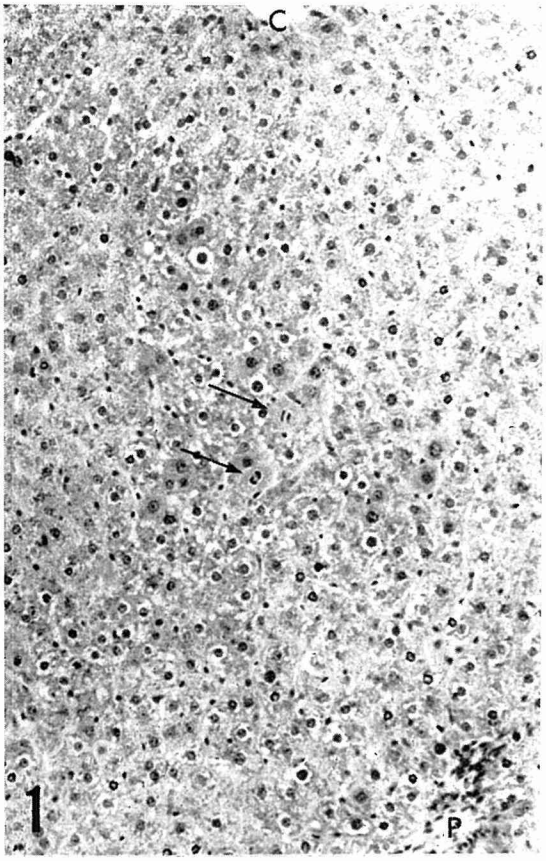
Photo. 8 In the 18th week. Hyperplastic nodule (HN). H-E. ×40.

Photo. 9 In the 24th week. Well differentiated trabecular hepatoma. H-E. ×40.

Photo. 10 In the 28th week. Carcinoma metastasized to the lung. H-E. ×40.

Photo. 11 Electron micrograph of basophilic cells (B) at the 16th week. Three basophilic cells are seen. ×5,200.

Photo. 12, 13 Higher magnification of Photo 11 ×1,100.
N: Nucleus. Mt: Mitochondria. G: Golgi apparatus. Mb: Microbody. Ly: Lysosome.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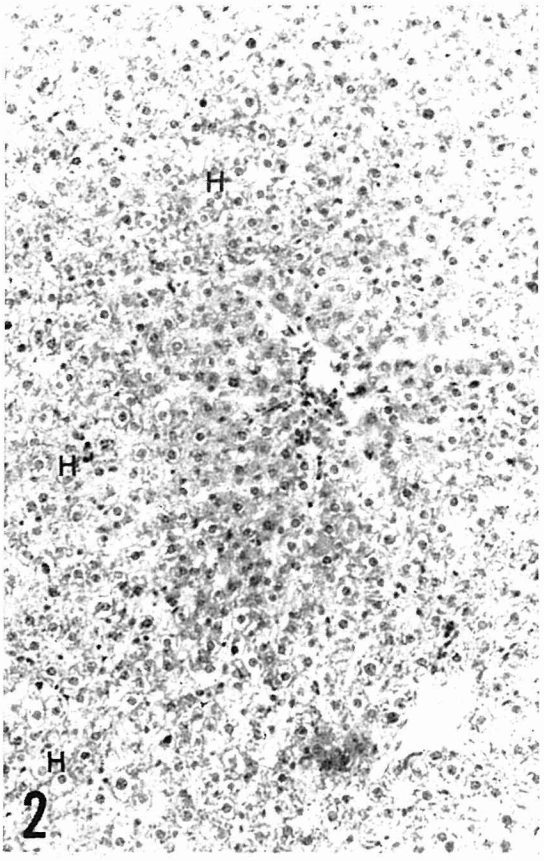
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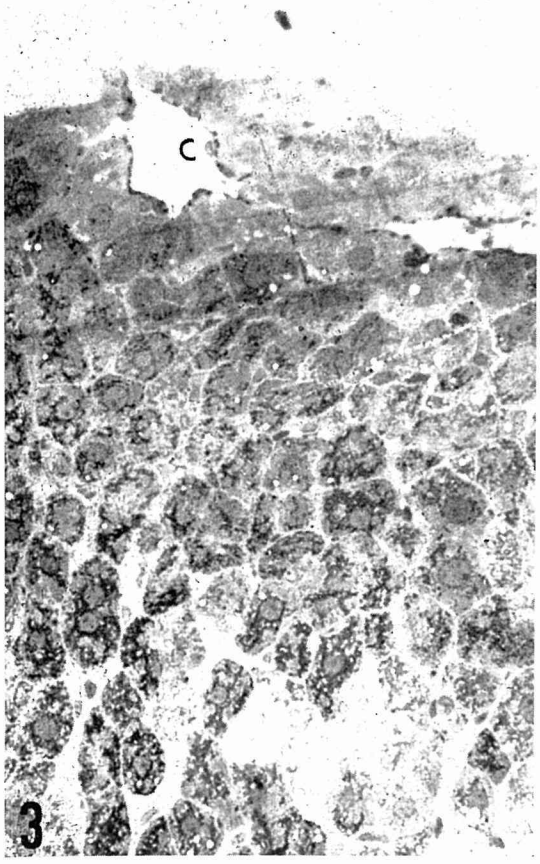
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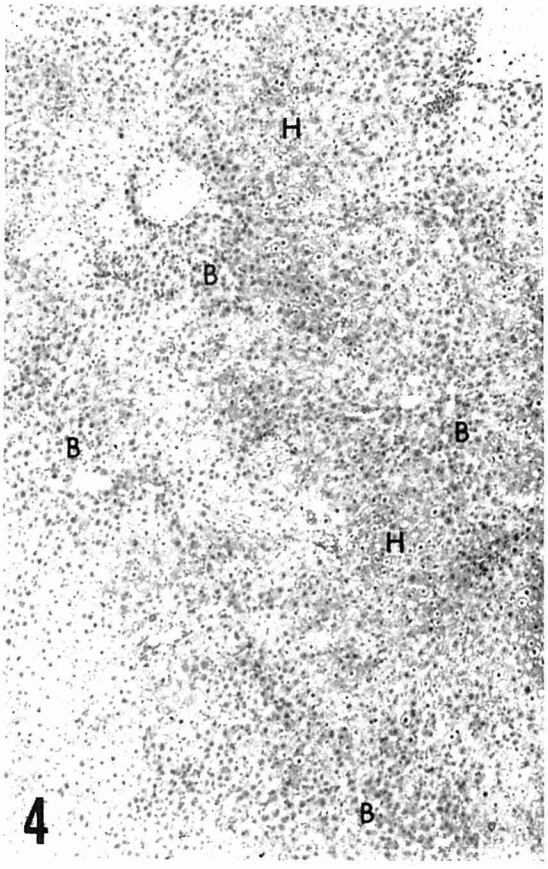
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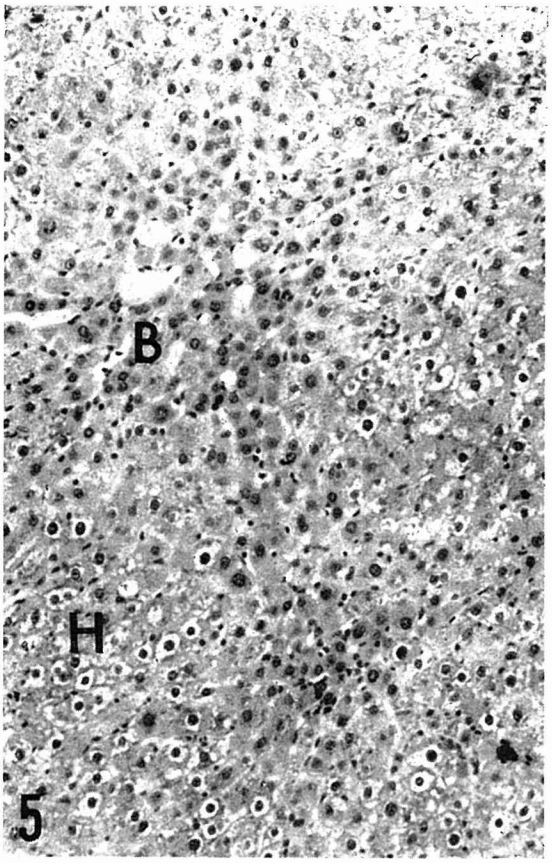
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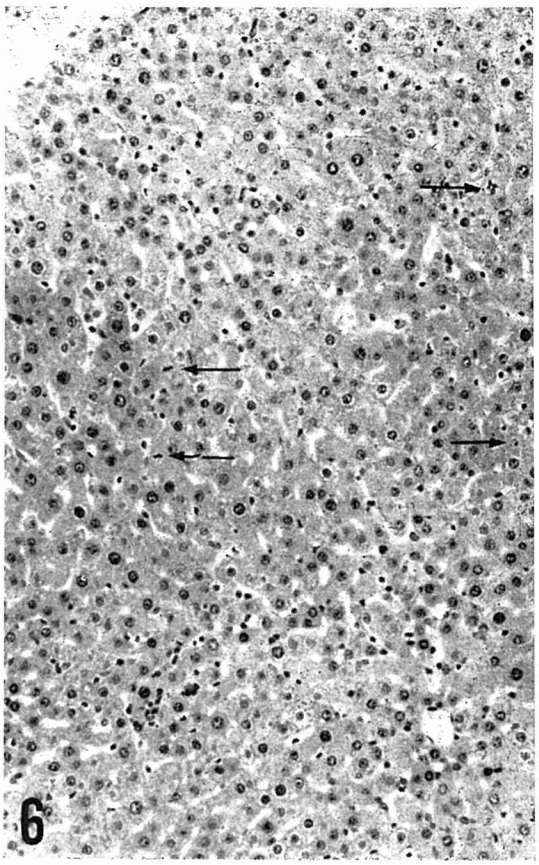
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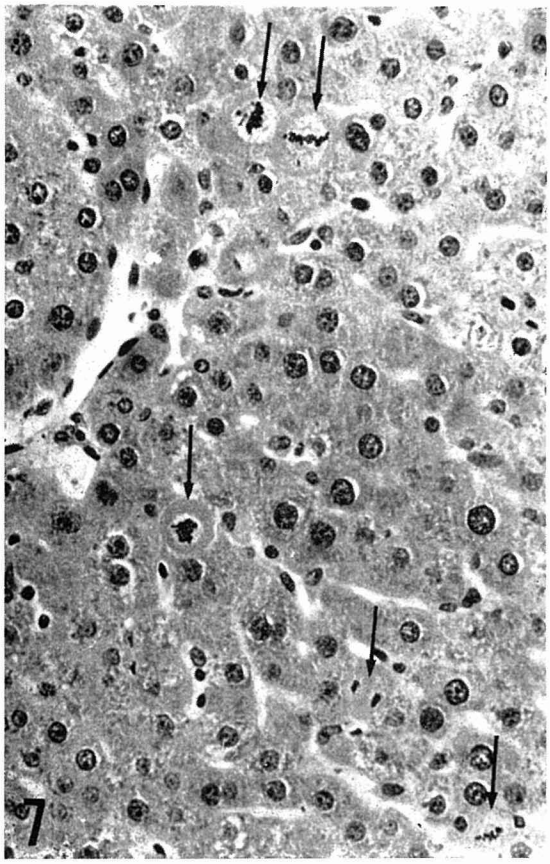
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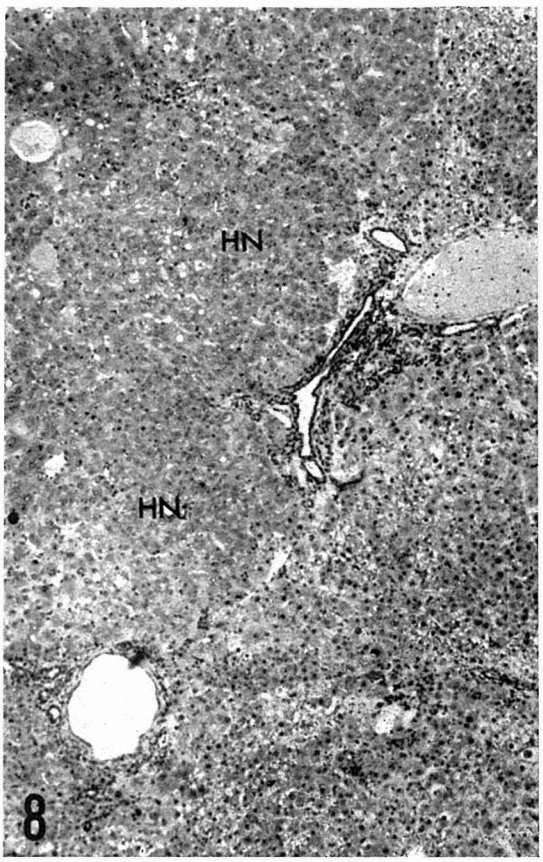
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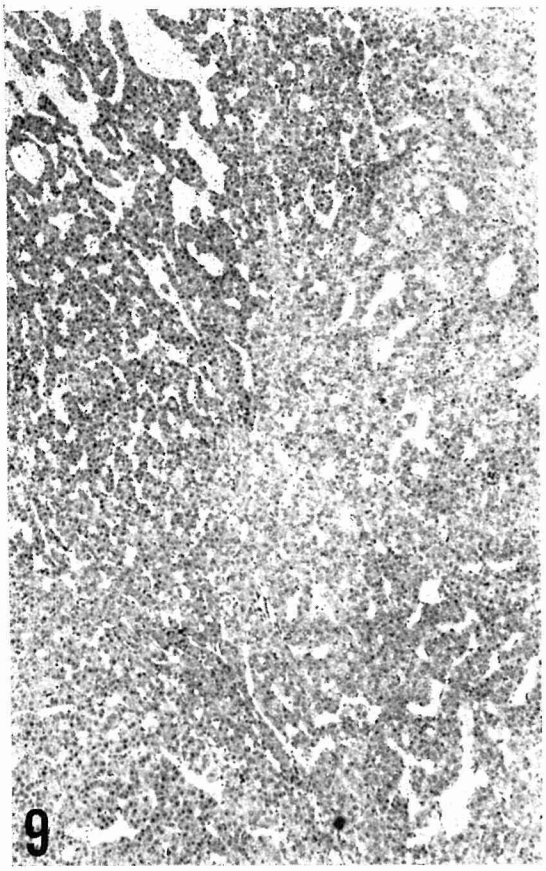

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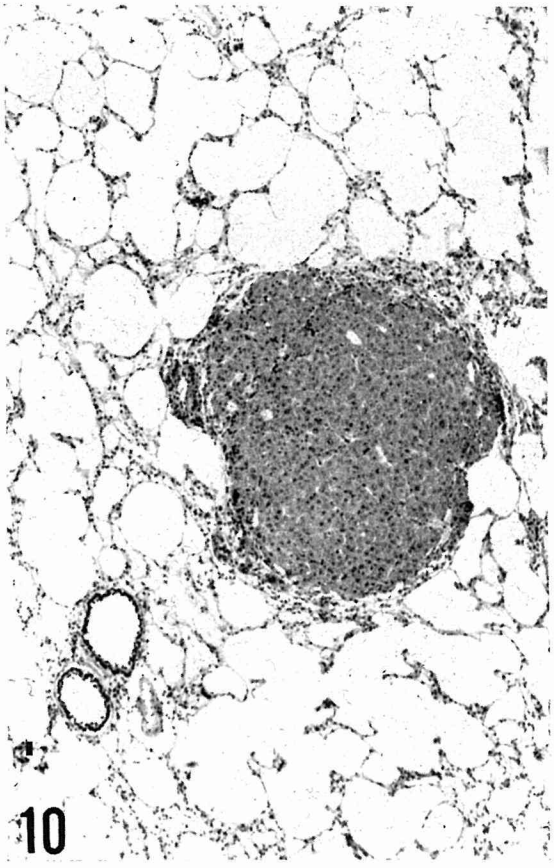

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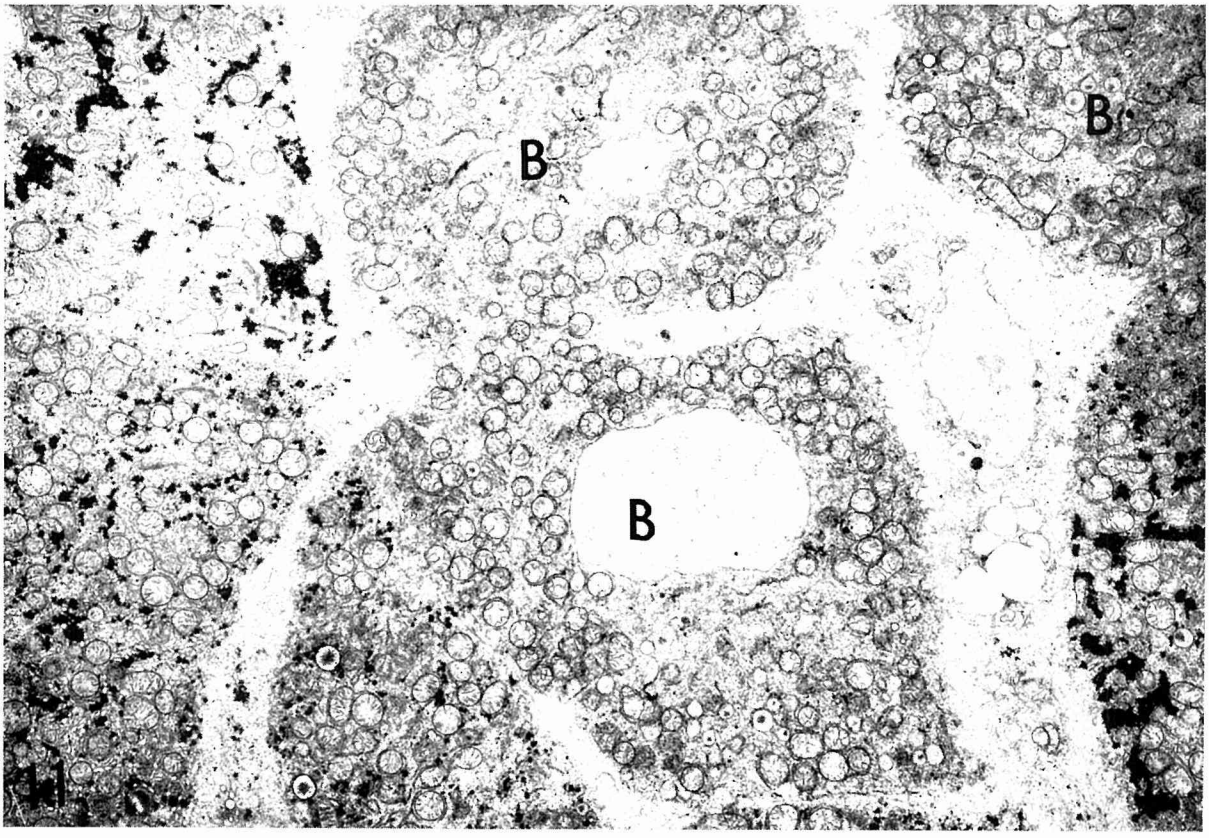

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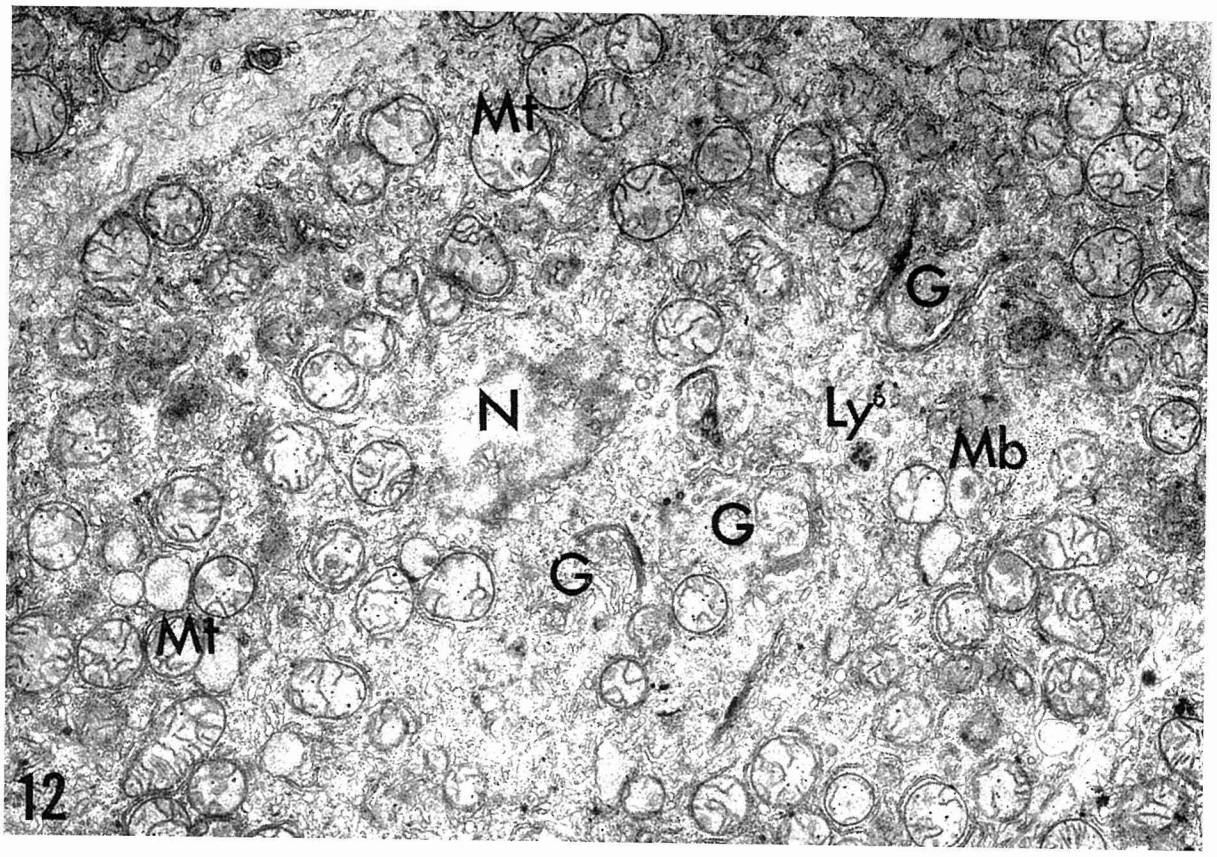
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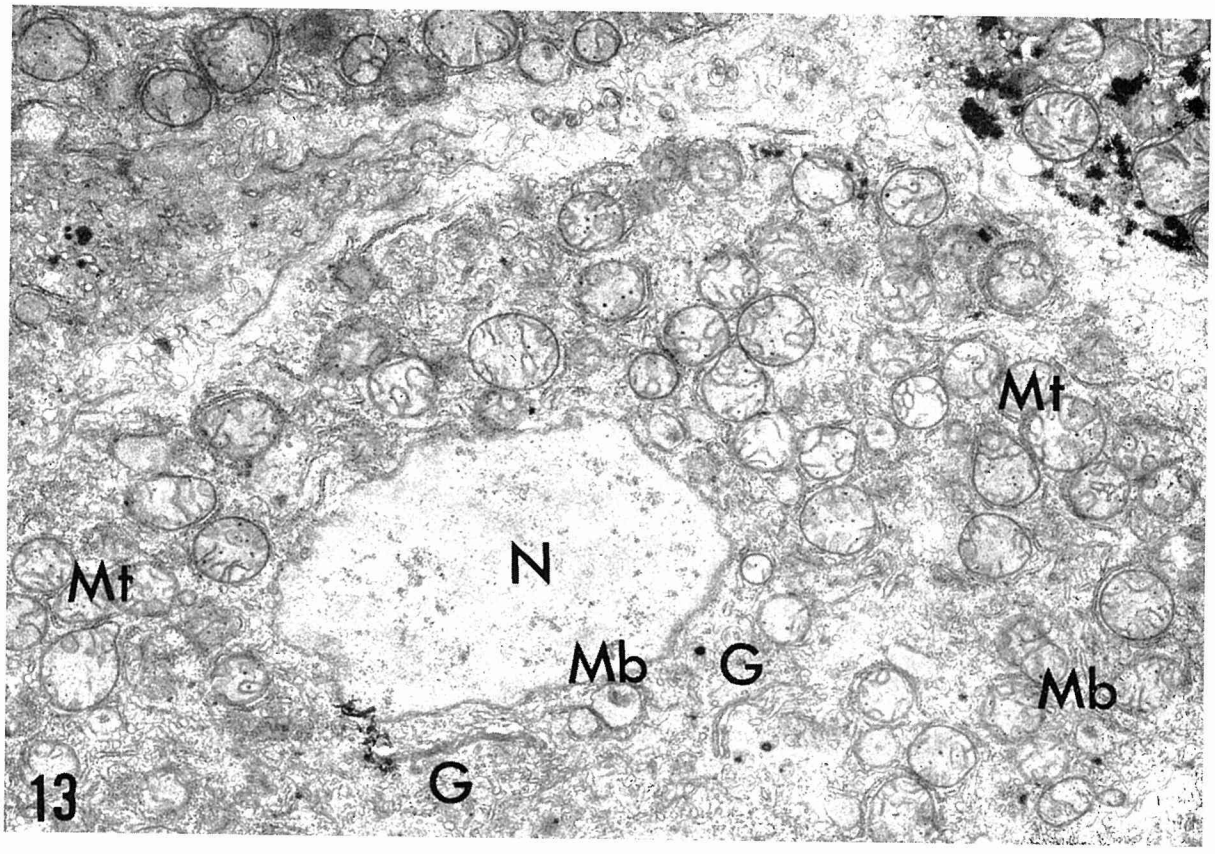
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