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ELECTRON-MICROSCOPIC EVALUATION OF THE HEPATOTOXICITY OF SELECTED HYDROCARBONS

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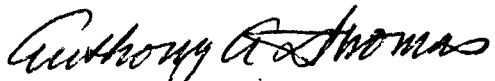

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20. ABSTRACT *(Continue on reverse side if necessary and identify by block number)* Morphometric analyses of livers of mice exposed to chlorobromodifluoromethane (H-1211), chlorobromomethane (H-1011), or phenobarbital were performed using electron-microscopic techniques. The data indicate that exposure to phenobarbital induced a significant increase in the surface area of smooth endoplasmic reticulum (SER). In contrast, neither H-1211 or H-1011 induced statistically significant increases in the surface area of SER. The ultrastructural appearance of the livers of animals exposed to both of the fluorocarbons was essentially normal although there was increased glycogen deposition in the H-1011 treated livers.

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PREFACE

This research was initiated by the Toxicology Branch, Toxic Hazards Division, Aerospace Medical Research Laboratory under Project 2312, Task V1. Work was performed under Contract F33615-76-C-0504, November 1975 to January 1977, by the Rush-Presbyterian-St. Luke's Medical Center, Chicago, Illinois 60612.

The principal investigator was Ronald S. Weinstein, M.D. Kenneth C. Back, Ph.D., was contract monitor for the Aerospace Medical Research Laboratory.

INTRODUCTION

Halogenated hydrocarbons are metabolized in the smooth endoplasmic reticulum (SER) of mammalian liver. Many halogenated hydrocarbons are noted for their hepatotoxicity (Browning, 1965; Recknagel, 1967; Weinstein et al; 1972). It is widely held that halogenated hydrocarbons are metabolized to free radicals which cause lipoperoxidation of hepatic intracellular membranes and, by this mechanism, produce hepatic necrosis (Recknagel, 1967). These effects can be potentiated by increasing the capacity of the liver to metabolize halogenated hydrocarbons, for example, by inducing proliferation of hepatic smooth endoplasmic reticulum (SER) phenobarbital (Smuckler, 1969).

In this quantitative electron microscopy study, we determined if two halogenated hydrocarbons of interest to the U.S. Air Force, Halon-1211 (H-1211) and Halon-1011 (H-1011), can induce proliferation of SER. Phenobarbital, a known SER inducer, was used as a positive control. Employing the intercept counting method developed by Weibel (1969), we determined the surface area of SER membranes in the livers of Carworth CF-1 mice which were intermittently exposed to H-1211 and H-1011 in inhalation chambers.

MATERIALS AND METHODS

Inhalation experiments were performed at the Aerospace Medical Research Laboratories, Wright Patterson Air Force Base. Carworth CF-1 male mice, weighing 18-25 grams, were used in all experiments. Ninety randomly bred male mice were selected for each experimental group. Of these mice, the organs of 15-20 were examined by light microscopy and 10 of these by electron microscopy. Mice were placed in inhalation chambers containing either Halon-1211 (bromochlorodifluoromethane) at a concentration of 15%; or Halon-1011 (bromochloromethane) at a concentration of 0.2 - 0.4%. The mice were exposed to the Halon for five hours daily on each of three consecutive days. Eighteen hours following the third inhalation exposure, mice were sacrificed by cervical dislocation. Two control groups were examined. The first control group received physiological saline I.P. on each of three consecutive days. A positive control group of mice received sodium phenobarbital, 80mg/per kilogram, I.P., daily for three days. Mice in each control group were sacrificed eighteen hours following the final injection. All mice received lab chow and water at ad lib, except during the exposure periods.

Following sacrifice, a complete autopsy was performed on each of the mice. Body weights and liver weights were recorded. Samples of heart, liver, lung and kidney were processed for routine histopathological examination. For electron microscopy, samples from livers of ten animals in each group were examined. Five to 10-1 mm^3 tissue blocks from each liver were prepared for thin section electron microscopy. Additional blocks were used for freeze-fracture electron microscopy. Blocks of liver were fixed by immersion in 1% osmic acid in phosphate buffer, pH 7.4. All samples for thin section electron microscopy were dehydrated by serial passage through graded ethanol solutions and embedded in EPON 812. Thin sections were cut on diamond knives, stained with uranyl acetate and lead citrate and photographed in either a RCA-3 or a Philips EM-300 electron microscope.

Livers from a minimum of five mice in each group were processed for morphometric analysis according to the methods of Weibel (1969). For morphometric analysis, livers were bread-loafed. Each slice was minced into many blocks. The blocks were randomized and five to seven blocks were selected for further processing. A plastic section was cut from each block for light microscopy and another for thin section electron microscopy. Five microscopic fields were photographed at a magnification of 20,540X in the electron microscope from each sample block. For each animal group, a minimum of 125 electron micrographs was analyzed as described by Weibel (1969). Separate saline- and phenobarbital control groups were used in the analysis of each of the Halons. Therefore, morphometric analyses were performed on a total of 750 electron micrographs.

For this study, surface area of SER and RER were related to a unit volume (CM^3) of hepatocyte cytoplasm. (It was determined experimentally that the partial volume occupied by hepatocyte cytoplasm is not significantly different for all experimental and control groups). In order to determine the surface area (SV) of SER and the surface area of RER per CM^3 of cytoplasm, electron micrographs were viewed in a Weibel microprojection table equipped with 20x20 cm. test lattice containing 100 test points. Intercept counts and point counts were recorded on a differential counter. Surface areas and partial volumes were calculated as described by Weibel (1969). Data reduction was done on a Hewlett-Packard 9830A programmable calculator equipped 4K memory.

RESULTS

Liver and Body Weights

Liver and body weight data for the mice in the Halon-1211 experiment and the Halon-1011 experiment are summarized in Tables 1 and 2. Comparison of liver weight/per 100 g. body weight ratios demonstrates that there is a significant ($p < .02$) increase in the ratio for phenobarbital exposed mice in each experiment. The liver/body weight ratio for mice exposed to H-1211 is not significantly different from the control value. However, the liver/body ratio for H-1011 mice is significantly elevated ($p < .02$) above control values.

Histopathology

Light microscopic examination of hematoxylin and eosin stained paraffin sections of heart, lung and kidney of animals in all groups reveal essentially normal structure. However, livers of mice exposed to H-1211 are finely vacuolated. Oil Red O stains of frozen sections from these livers demonstrates the presence of small fat droplets.

Electron Microscopy of Livers

Saline-Control: Livers of mice in all groups were preserved. Figure 1 shows a portion of hepatocyte in the liver of a saline-control mouse. The level of tissue preservation is excellent. Mitochondria, rough endoplasmic reticulum (RER) and smooth endoplasmic reticulum (SER) appear normal. Intracellular glycogen is abundant in all control livers (Figure 1).

TABLE 1. LIVER AND BODY WEIGHTS IN MICE

FOLLOWING INHALATION OF HALON 1211[a]

	Liver weight[b] (g)	Body weight (g)	Liver weight/ 100g body weight
Group			
Control	1.58 (± .135)	19.6 (± 1.30)	6.82 (± .934)
Halon - 1211	1.23 (± .218)	23.3 (± 1.67)	6.02 (± 1.263)
Phenobarbital	1.77 (± .165)	22.1 (± 2.42)	8.13[c] (± 1.26)

a - each group consisted of 10 male mice

b - mean ± 1 standard deviation

c - value is significantly different from the control value at a 2% level of significance.

TABLE 2. LIVER AND BODY WEIGHTS IN MICE

FOLLOWING INHALATION OF HALON 1011[a]

	Liver weight[b] (g)	Body weight (g)	Liver weight/ 100g body weight
Group			
Control	1.34 (± 0.106)	23.3 (± 1.67)	5.77 (± .28)
Halon - 1011	1.39 (± 0.1642)	20.6 (± 1.57)	6.79[c] (± .48)
Phenobarbital	1.55 (± 0.158)	22.1 (± 2.42)	7.07[c] (± .41)

a - each group consisted of 10 male mice

b - mean ± 1 standard deviation

c - value is significantly different from the control value at a 2% level of significance.

TABLE 3. LIVER ENDOPLASMIC RETICULUM SURFACE DENSITIES FOLLOWING INHALATION OF HALON 1211[a]

Group	S_v SER (m^2/cm^3)[a,b]	S_v RER (m^2/cm^3)
Control	2.61 (± .426)	4.60 (± 1.42)
Halon - 1211	3.01 (± .548)	2.93[d] (± .827)
Phenobarbital	4.83[c] (± 1.26)	2.07[c] (± .996)

a - m^2 of membrane per cm^3 of hepatocyte cytoplasm.

b - mean value ± 1 standard deviation

c - value is significantly different from the control value when tested by the students ± test at a 1% level of significance

d - value is significantly different from the control value at a 5% level of significance.

TABLE 4. LIVER ENDOPLASMIC RETICULUM SURFACE DENSITIES

FOLLOWING INHALATION OF HALON 1011[a]

	S_v SER (m^2/cm^3)[a,b]	S_v RER (m^2/cm^3)
Group		
Control	2.04 (± .329)	2.17 (± .313)
Halon - 1011	2.40 (± .331)	1.43 (± .133)
Phenobarbital	3.73[c] (± .372)	1.58 (± .2704)

a - m^2 of membrane per cm^3 of hepatocyte cytoplasm.

b - mean value ± 1 standard deviation

c - value is significantly different from the control value when tested by the students ± test at a 1% level of significance

d - value is significantly different from the control value at a 5% level of significance

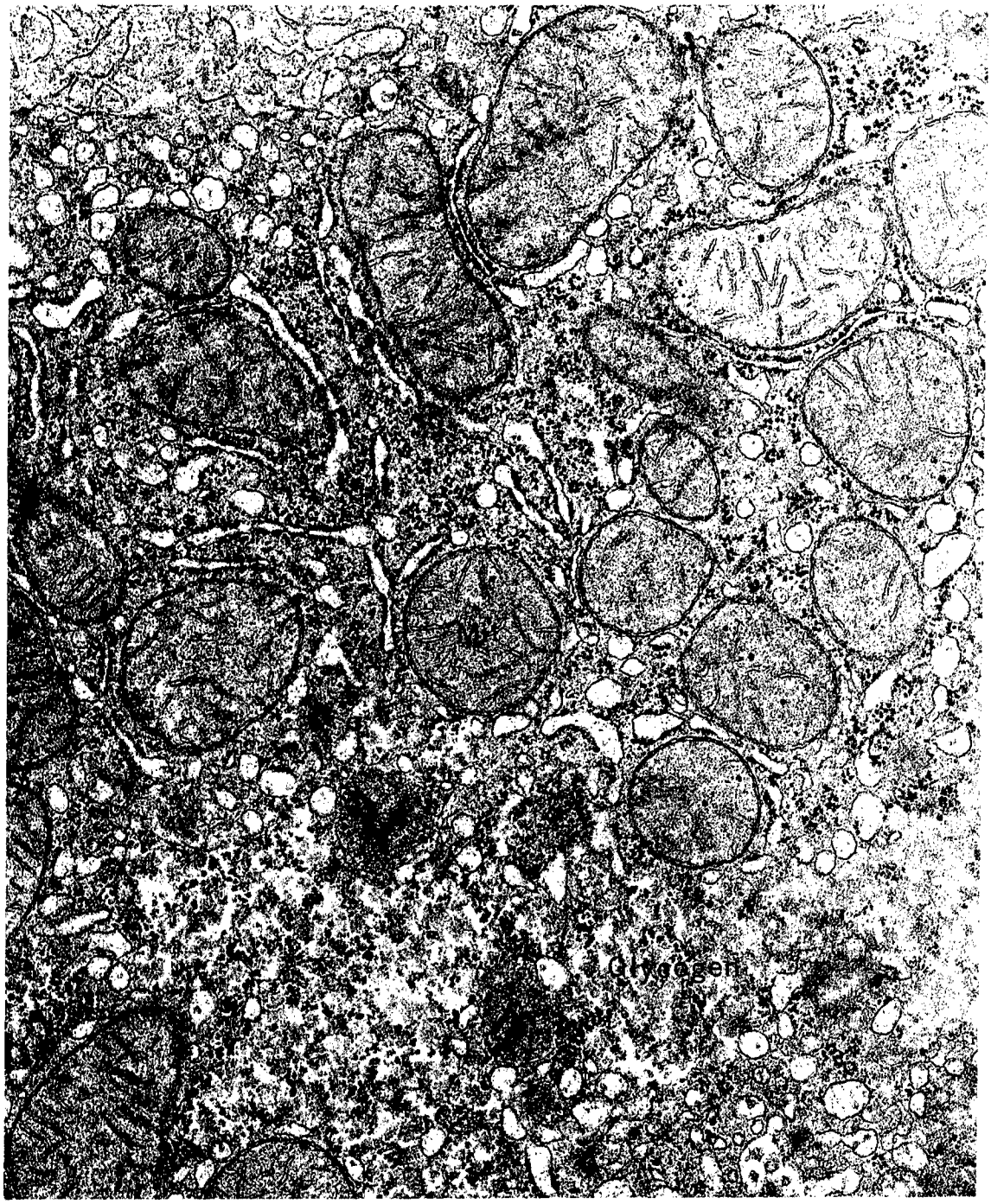

Figure 1. ELECTRON MICROSCOPY OF A HEPATOCYTE IN A SALINE-CONTROL MOUSE. SER, RER, and mitochondria (Mit) are well preserved. Glycogen is abundant. X 32,000.

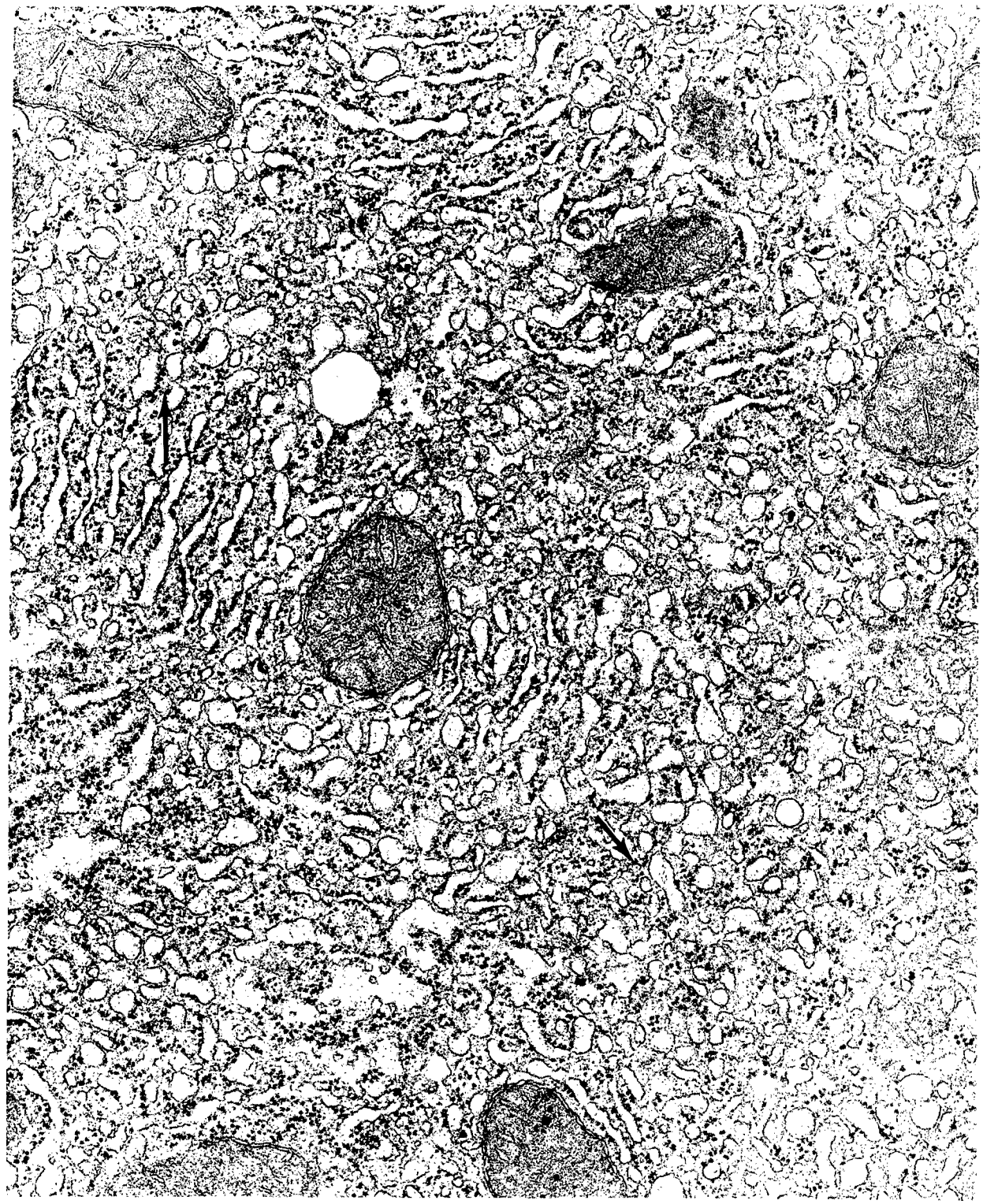

Figure 2. HEPATOCYTE IN A POSTIVE CONTROL MOUSE FOLLOWING PHENOBARBITAL INJECTION. SER (arrows) is actively proliferating. X 32,000.

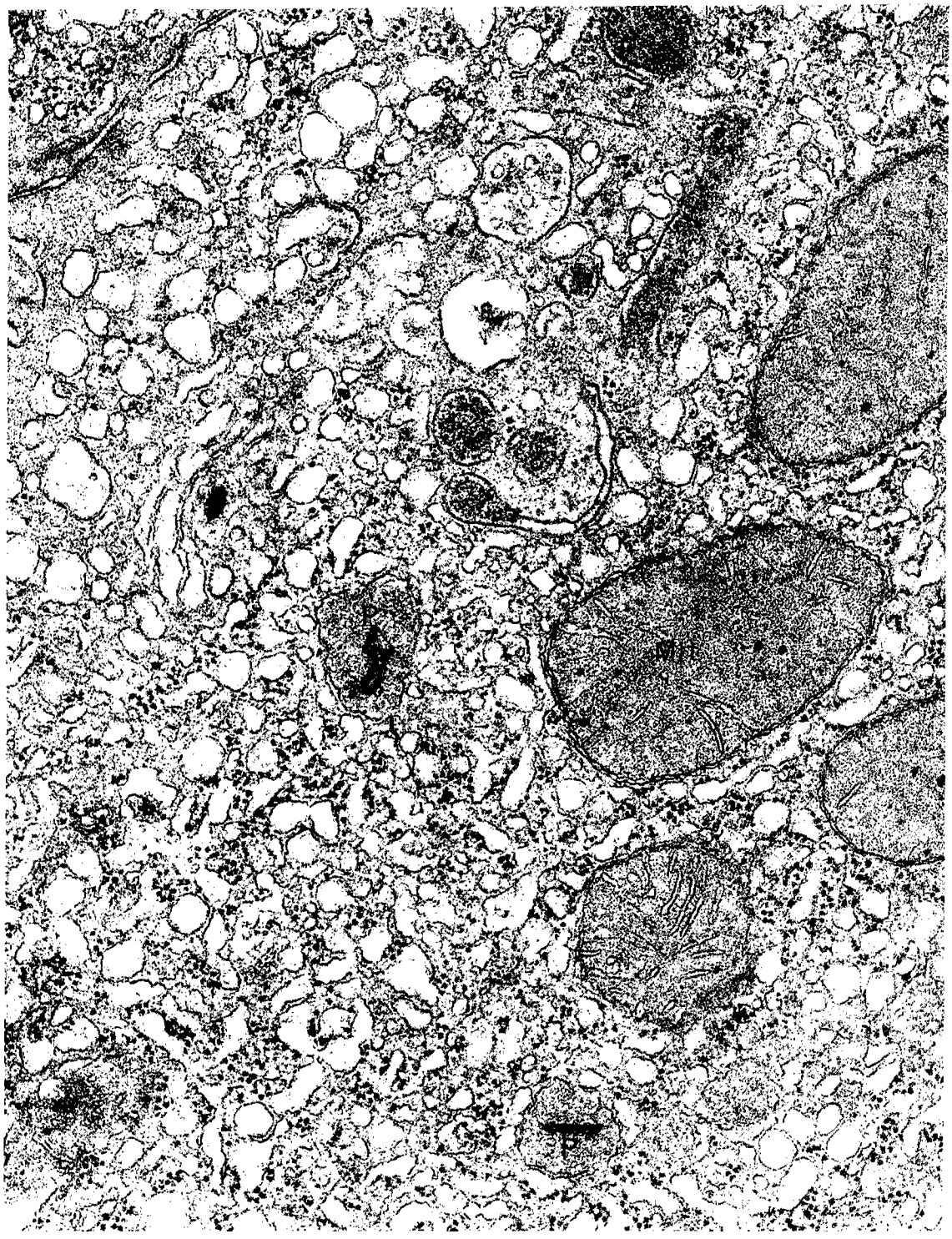

Figure 3. SER PROLIFERATION IN A HEPATOCYTE OF A PHENOBARBITAL CONTROL. Mitochondrial (Mit) and peroxisomes (P) appear normal. X 38,000.

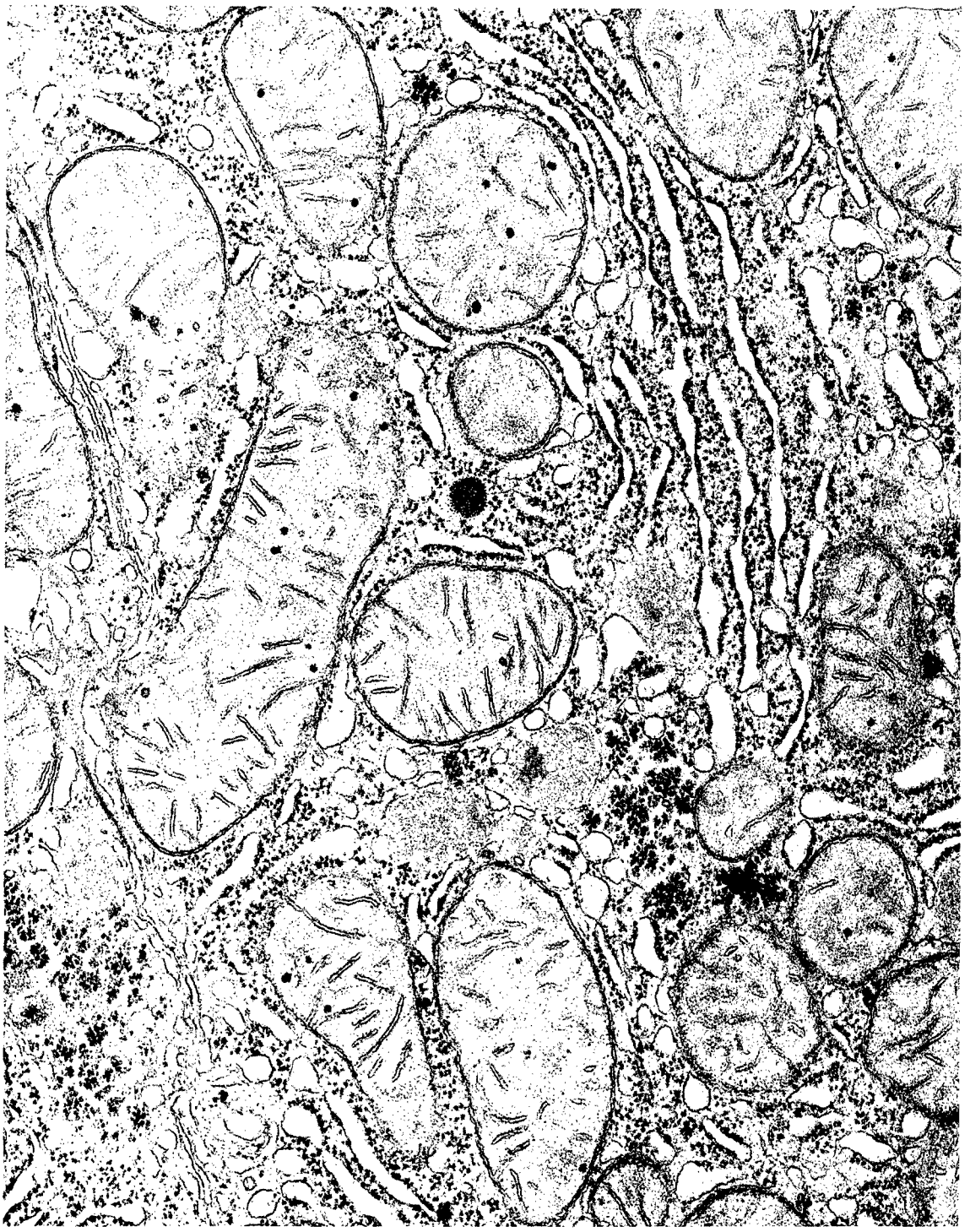

Figure 4. MOUSE HEPATOCYTE FOLLOWING H-1211 INHALATION. Mitochondria, RER and SER appear normal. X 34,000.

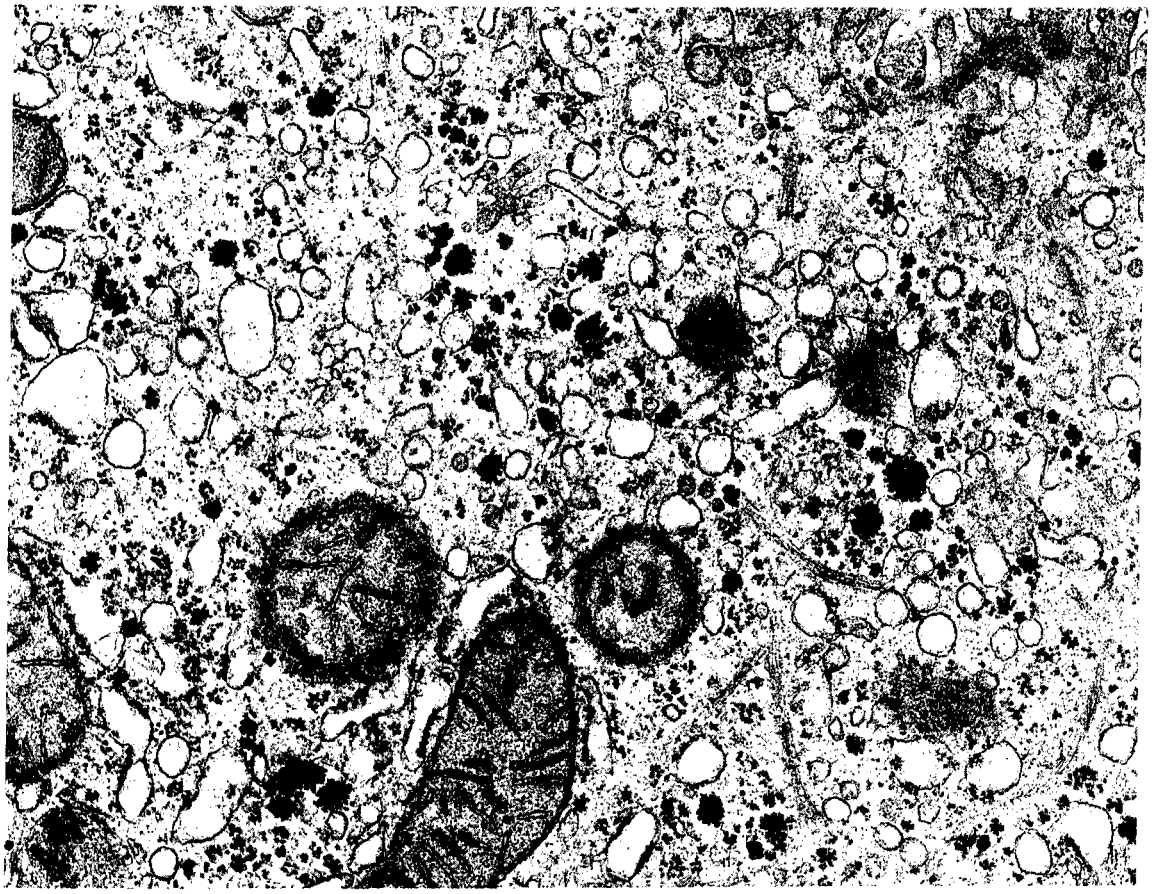

Figure 5. SER AND GLYCOGEN IN A HEPATOCYTE FOLLOWING INHALATION OF H-1211. SER shows no evidence of proliferation. X 32,000.

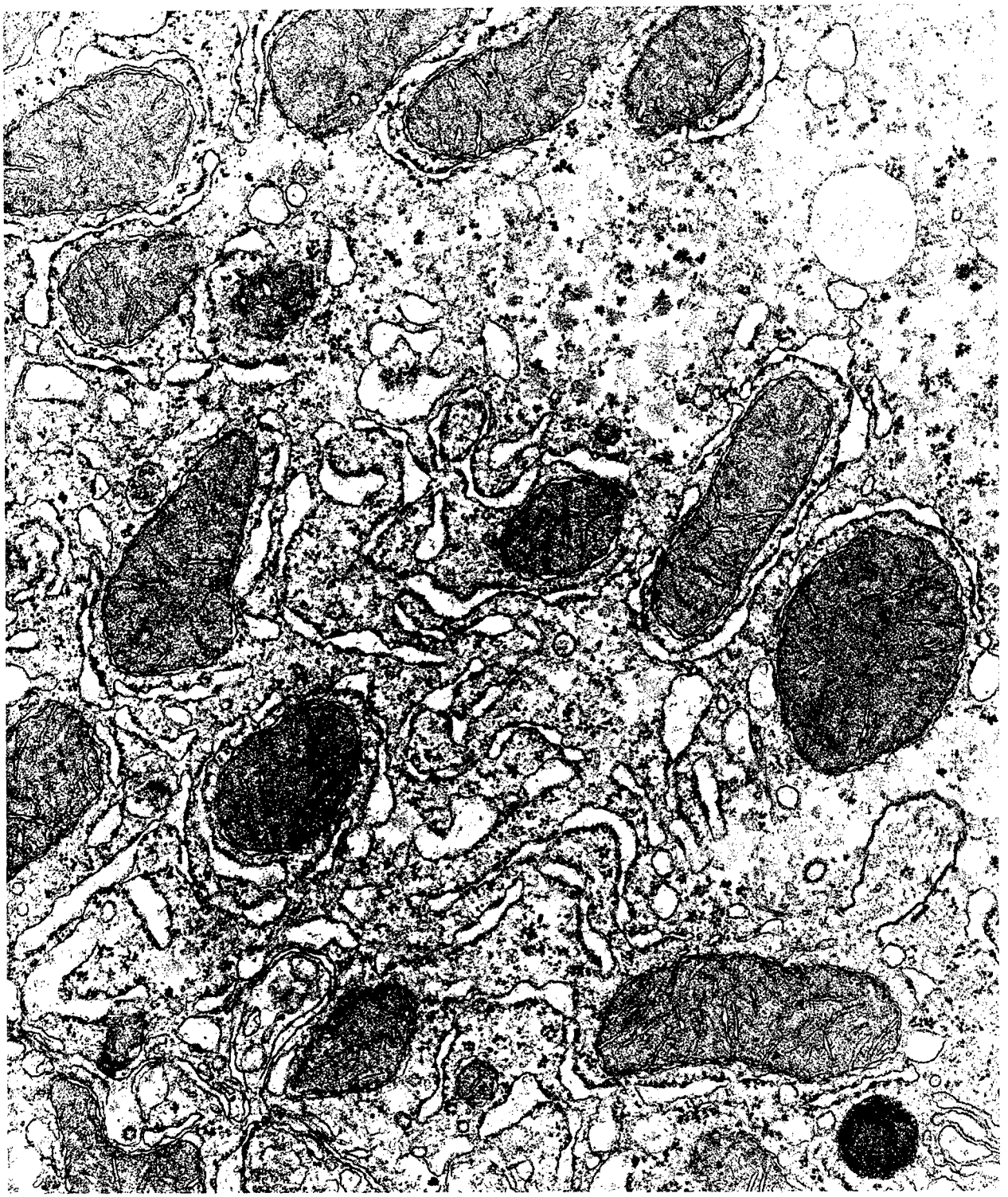

Figure 6. CYTOPLASM OF A MOUSE HEPATOCYTE FOLLOWING H-1011 INHALATION. Mitochondria (Mit) and ER appear unremarkable. X 31,000.

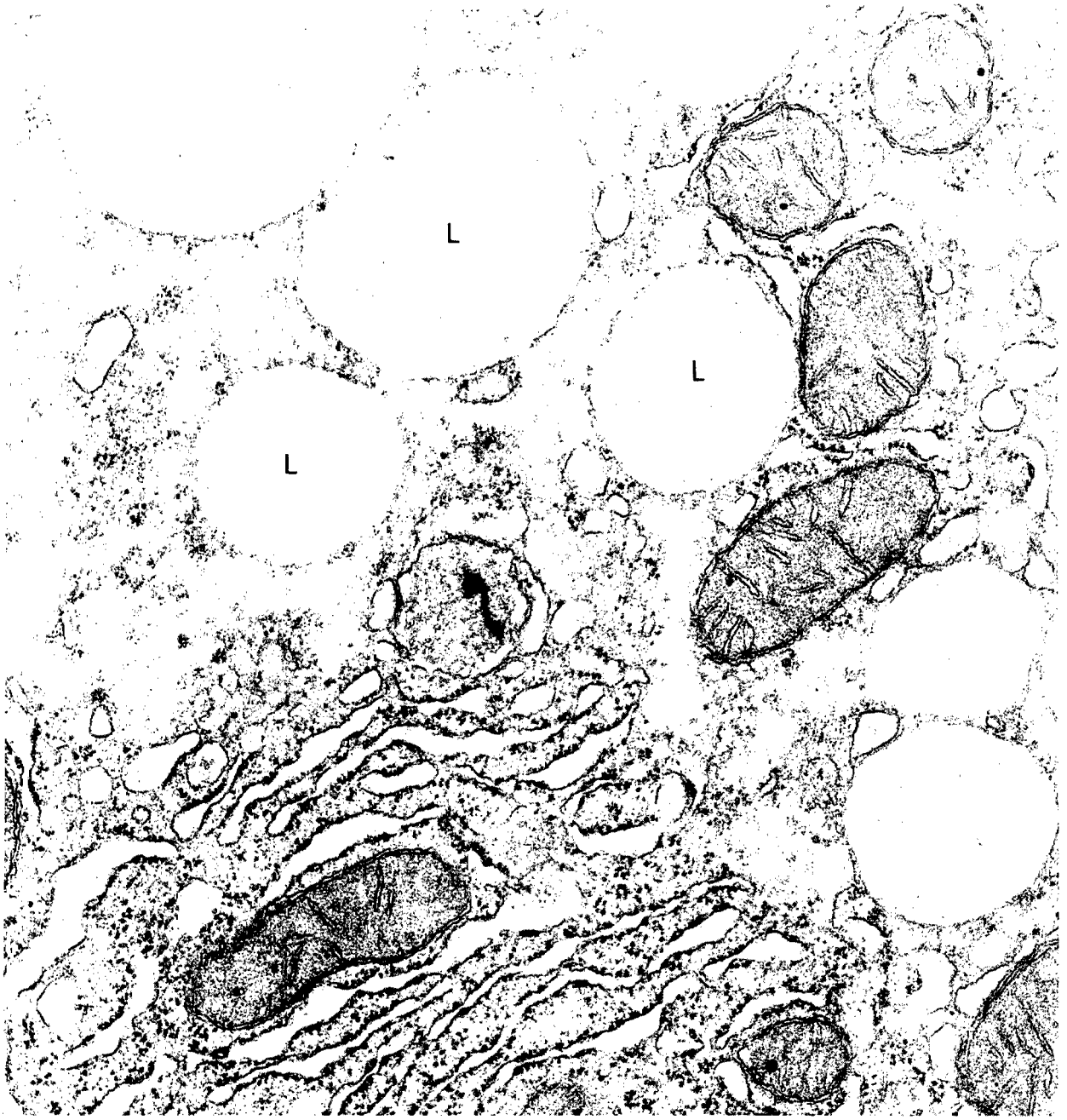

Figure 7. HEPATOCYTE OF A MOUSE FOLLOWING H-1011 INHALATION. Hepatic lipid droplets (L) are prominent. X 34,000.

Phenobarbitol-Controls: Livers of mice given phenobarbital I.P., show a striking proliferation of the SER throughout the hepatocytes. This is accompanied by a mild decrease in the amount of RER (Figure 2 & 3). All other organelles appear normal.

Halon-1211: The ultrastructure of hepatocytes in Halon-1211 exposed mice is essentially unremarkable. There is no apparent increase in the quantity of SER (Figure 5 & 6). Glycogen is abundant in the cells and the mitochondria appear entirely normal.

Halon-1011: Livers from mice exposed to Halon-1011 are indistinguishable from the liver of the Halon-1211 exposed mice (Figure 7) except for the presence of many small lipid droplets within the cytoplasm.

Quantitative Electron Microscopy: Morphometric analyses of liver confirm that there is a highly significant ($p < .01$), greater than 80% increase in the amount of SER in the phenobarbital-exposed positive control mice. This increase in SER can account for the significant increase in liver weights in these positive controls. Surface area of SER for the H-1211 (TABLE 3) and H-1011 (TABLE 4) mice are not significantly different from control values. The increase in liver weights for the H-1011 mice can be accounted for on the basis of an increase in hepatic fat, as was demonstrated by both light and electron microscopy.

DISCUSSION

SER membranes in the hepatocytes of liver are of interest in industrial toxicology since these membranes contain cytoplasmic mixed function oxidase systems which are highly cross reactive. The SER participate in many metabolic pathways. It is the site of hepatic drug detoxification. Further, SER membranes participate in steroid and lipid metabolism, are responsible for the activation of some carcinogens, and they may contribute to the hepatotoxicity of certain classes of compounds such as the halogenated hydrocarbons. In the current study, interest has been focused on the influence of halogenated hydrocarbons on SER proliferation. Two compounds were examined, Halon-1211 and Halon-1011. Quantitative electron microscopy techniques (so-called morphometric techniques) were used to quantitate the surface area of SER membranes in the hepatocytes of mice following intermittent inhalation of their halogenated hydrocarbons. Phenobarbital, a drug which is known for its ability to induce a striking proliferation of SER membranes, was used as a positive control.

The doses of the Halons used in these experiment are relatively high, compared to those which humans might be expected to be exposed to in an industrial setting. It is therefore pertinent to note that autopsy revealed little evidence of injury to heart, lung, liver or kidney following multiple exposures. The only evidence of toxic injury in either experimental group was in livers of mice exposed to Halon-1011 at a concentration of 0.4%, for five hours daily on each of three consecutive days. Liver weight was mildly increased and microscopic examination demonstrated mild fatty change. Other than these findings, light microscopy revealed essentially normal histology in all organs examined. Quantitative electron microscopy studies showed that

neither H-1211 or H-1011 produced a statistically significant increase in SER. This is in contrast to the positive control mice which received I.P. injections of phenobarbital. In these mice, there was a striking intracellular proliferation of SER. This increase was not present in mice injected with saline solution. These essentially negative results support a hypothesis that Halons are not inducers of SER.

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