MORPHOMETRIC ANALYSIS OF THE ULTRASTRUCTURAL CHANGES IN RAT LIVER INDUCED BY THE PEROXISOME PROLIFERATOR SaH 42-348

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ABSTRACT

The changes occurring in hepatocytes of F-344 male rats during a 3-wk treatment with a hypolipidemic agent, 1-methyl-4-piperidyl-*bis*[*p*-chlorophenoxy]acetate (SaH 42-348), have been evaluated by morphometric and biochemical methods. The twofold increase in liver weight resulted from a significant increase in hepatocyte cytoplasm as well as a moderate increase in the number of liver cells. The peroxisome population and SER played an overwhelming part in the hypertrophy of hepatocytic cytoplasm. The relative volume and the surface density of peroxisomes increased ninefold and sevenfold, respectively. The increase in the collective peroxisome volume resulted from an increase in both the number and the average volume of peroxisomes. The SER also demonstrated a substantial increase in these values. The relative volume and surface density of mitochondria were not significantly altered in comparison to controls, while these values for RER decreased onefold. Studies on the lobular distribution of cytoplasmic organelles before and during treatment revealed that the relative volume and surface density of peroxisomes and SER increased from periportal to centrilobular cells of the hepatic lobule, whereas mitochondrial values decreased from periportal to centrilobular cells. The RER values were fairly constant in different parts of the hepatic lobule. The increase in peroxisome and SER volume and surface area was first evident within the first 3 days of SaH 42-348 treatment and these values continued to increase, reaching a steady state within 2 wk. The time course of increase in catalase and carnitine acetyltransferase activities correlated with the morphometric data on the peroxisomes. After cessation of SaH 42-348 treatment, the peroxisome values decreased rapidly within the first 3 days and reached control levels within 1 wk. Moderate reduction in SER values occurred after withdrawal of the drug, but these values remained higher than controls even after 2 wk, suggesting that the reduction in the amount of circulating peroxisome proteins may result in empty SER channels. On the 4th day of drug withdrawal a significant increase in the relative volume and surface density of lysosomes was

observed, suggesting that these organelles may play some part in the removal of cellular membranes. However, the rapid reduction in peroxisome values after SaH 42-348 withdrawal appears to be due to cessation of enhanced peroxisome protein synthesis.

The function of mammalian peroxisomes (microbodies, microperoxisomes) has yet to be elucidated. Rhodin first described these organelles in the renal proximal tubule as a single-membrane-limited cytoplasmic constituent with a fine granular matrix (39). Further studies have since demonstrated peroxisomes in several animal tissues, protozoans, and plants. The content of mammalian peroxisomes was shown by de Duve and co-workers to include catalase and several oxidative enzymes such as urate oxidase, D-amino acid oxidase, L-α-hydroxy acid oxidase, and isocitrate dehydrogenase (21). More recently, the activities of carnitine acetyltransferase, NAD^+:α-glycerol phosphate dehydrogenase, and NADH-cytochrome *c* reductase have also been reported in peroxisome fractions (10, 15, 26). One area of study which may help to elucidate the function of peroxisomes and their contents is the use of certain hypolipidemic drugs which have been shown to induce proliferation of hepatic peroxisomes.

In 1964, Duncan and co-workers reported that the hypolipidemic drug, clofibrate, caused a marked hepatomegaly in rats (11). The following year, Hess and co-workers found that the hepatomegaly arose from a striking proliferation of peroxisomes in the liver cells (17). A proliferation of the smooth endoplasmic reticulum (SER) also contributed to the hepatomegaly, but to a lesser extent. Methyl clofenapate, nafenopin, and SaH 42-348 (1-methyl-4-piperidyl *bis*[*p*-chlorophenoxy]acetate), which are hypolipidemic analogues of clofibrate, have also been found to induce hepatomegaly with a proliferation of peroxisomes (34, 35, 37). In addition, a few other compounds which are structurally unrelated to clofibrate have been shown to induce both hypolipidemia and peroxisome proliferation (18, 20, 36). On the basis of this relationship, it has been suggested that peroxisomes may be related to lipid metabolism (36).

The increase in peroxisome proliferation after treatment with these drugs was shown to be accompanied by an increase in catalase activity (27, 37), the marker enzyme for peroxisomes (21). An increase in the activities of carnitine acetyltransferase (CAT) and NAD^+:α-glycerol phosphate dehydrogenase also occurs upon treatment with these compounds (19, 27, 37).

The purpose of this study is to describe quantitatively the structural events which lead to hepatomegaly, on the basis of a morphometric analysis (47, 48) of hepatocytes in rats treated with SaH 42-348. These structural alterations in liver cells are correlated with the changes in hepatic catalase and carnitine acetyltransferase activities. Also included in this report are studies on the effect of withdrawal of SaH 42-348 on the reversal of peroxisome proliferation, in order to assess the role of the lysosomal system in the removal of peroxisomes. Preliminary results of this study have been presented elsewhere (28).

MATERIALS AND METHODS

Animals

The animals used in these studies were male F-344 rats (Simonson Laboratories Inc., Gilroy, Calif.) weighing 130–160 g, which were housed in individual steel cages. SaH 42-348 (Sandoz Pharmaceuticals, Hanover, N. J.) was mixed with ground Purina rat chow at a concentration of 0.10% (wt/wt). Animals received food and water ad libitum; control animals received a similar diet without the drug.

Animals were fasted for 12 h and sacrificed under light ether anesthesia between 10:30 and 11:30 a.m. Treated animals (three per each interval) were sacrificed on days 1, 3, 6, 10, 14, and 21. After 21 days, the remaining animals were returned to the control diet and sacrificed on 2, 4, 8, and 14 days after withdrawal. Control animals were sacrificed on days 8, 17, and 31 of the experiment.

Biochemistry

LIVER CATALASE: Livers were perfused with isotonic saline solution via the portal vein, removed, and weighed. A 5% homogenate in distilled water was prepared according to the method described previously (34) and the catalase activity determined spectrophotometrically at 25°C as described by Lück (25).

LIVER CARNITINE ACETYLTRANSFERASE: 20% homogenates of liver were prepared in a buffer containing 116 mM Tris-HCl, 2.5 mM EDTA, and 0.25 M sucrose (pH 8.0). Supernates were prepared as described previously (27) and assayed spectrophotometrically for CAT with the thioacceptor DTNB [5,5′-

dithio*bis*(2-nitrobenzoic acid)] as described by Markwell et al. (26).

LIVER SUCCINATE DEHYDROGENASE: Succinate dehydrogenase activity was measured by the spectrophotometric method of Bachmann and co-workers (1) on liver homogenates in 0.25 M sucrose.

CELL FRACTIONATION: Liver homogenates in 0.25 M sucrose were fractionated by differential centrifugation (19). Proteins were measured by the method of Lowry et al. (24).

Morphology

Samples of liver were fixed for 1 h at 4°C in 2% osmium tetroxide buffered with *s*-collidine to pH 7.4, and processed as described previously (44). For light microscopy, 1-μm sections were stained with toluidine blue and viewed in a Zeiss Ultraphot II microscope. Thin sections were stained with lead hydroxide and examined with a Hitachi Hu 11-C electron microscope at 50 kV. The magnification was calibrated regularly with a replicate diffraction grating (30×10^6 lines/in).

Morphometric Analysis

Morphometric analysis was performed essentially as described by Weibel et al. (47). At all stages of investigation, three blocks of tissue were randomly selected from each animal. Sections from these blocks were then examined at three stages of magnification.

STAGE I: 1-μm sections stained with toluidine blue were examined with a Zeiss Ultraphot II microscope at a magnification of 1,130. A grid (10 cm × 8 cm) containing cross-hatchings spaced 1 cm apart (63 points of intersection) was drawn on a glass plate and inserted in place of the normal viewing plate. 20 fields each were examined from groups sacrificed on 21 days of treatment, 14 days after withdrawal from treatment, and from control animals (blocks from one animal were selected from each of the three control groups).

STAGE II: Thin sections were examined under the electron microscope at a magnification ranging from 4,030 to 4,140. Fields of hepatocyte cytoplasm were selected in the random manner suggested by Weibel et al. (47) and those free of artifacts were photographed. Five photographs were taken per animal and 10 in × 8 in prints were produced from the negatives. The magnification of prints (~3) was determined from each negative in order to correct for shrinkage and other alterations from the photographic processing. The final magnification at this stage was approximately 12,000. Prints were analyzed by the multipurpose test system of Weibel et al. (47) in which 84 lines (Z = 1.5 cm) were used, giving 168 counting points per field. Point counting was used to estimate the relative volume of cytoplasmic organelles and intersections of test lines with membranes were counted to estimate the surface density of the respective limiting membranes. All organelles were analyzed at this lower magnification in order to minimize variation due to possible nonrandom distribution of certain organelles in hepatocytes.

STAGE III: Additional fields were analyzed at a higher magnification (final magnification ~23,000) to increase the accuracy of counts on membranes of low resolution (i.e. SER, RER, and Golgi apparatus). The procedure was otherwise identical to that of stage II, with five prints per animal being analyzed. No significant difference was found in the values determined at these two stages, and values were pooled for the final results. Therefore, a total of 30 prints per interval for treated and withdrawn animals and 20 prints per interval for control animals were evaluated.

Analysis of Lobular Distribution

The lobular architecture of the tissue was determined on toluidine blue-stained 1-μm sections. Those demonstrating clearly definable centrilobular or periportal vessels were sectioned and stained with lead for examination under the electron microscope. The centrilobular and periportal fields were selected from three rows of hepatocytes adjacent to the respective vessels. This was based on Loud's observation (23) that the most significant heterogeneity of liver cells was found in these areas. Midzonal fields were selected from cells at least seven rows away from either group of vessels. Photographs were taken of cell cytoplasm and analyzed at the magnification of stage III. In all, 15 prints for each area were analyzed on control and 21 day-treated animals.

Calculations and Statistics

All the calculations of morphometric analysis were as described by Weibel et al. (47). To determine the absolute values of volume (cm^3/liver) and surface area (m^2/liver) the density of liver was assumed to be 1.05 g/cm^3 as reported by Weibel et al. (48). Values calculated during the treatment and withdrawal period were compared to the pooled values of the three control groups. Levels of significance were determined by using Student's two tail *t*-test. To compare the centrilobular, midzonal, and periportal values, the three groups were tested for nonequality by the ANOVA one-way analysis as described by Dixon and Massey (9). When nonequality was found ($P < 0.05$), the values that were not equal were determined by Duncan's multiple range test (12).

RESULTS

Effect on Liver Size

On gross examination of rats treated with 0.10% SaH 42-348, the most noticeable alteration was the enlargement of the liver. The relative liver weight (RLW) of treated rats showed a rapid increase during the 1st wk of treatment (Fig. 1). A slight reduction in this trend occurred on day 10 and was followed by a further increase which

reached a plateau by the 2nd wk at twice the control level. Upon withdrawal of SaH 42-348 from the diet, the RLW returned to control levels within 14 days.

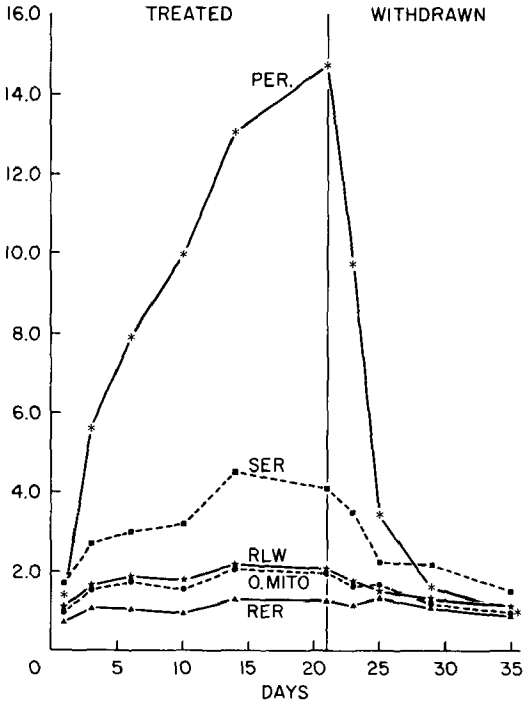

FIGURE 1 Total liver value for the surface area of hepatocyte organelles in F-344 rats receiving 0.10% SaH 42-348 expressed as ratio to control values and compared to the ratio of relative liver weight in treated animals to control animals. *PER*, peroxisomes; *SER*, smooth endoplasmic reticulum; *RER*, rough endoplasmic reticulum; *O MITO*, outer mitochondrial membrane; *RLW*, relative liver weight.

The changes in hepatocyte size and number were evaluated by morphometric analysis of semithin sections. It was found that the number of total liver nuclei increased 1.4 times over control values (Table I). The average hepatocyte volume, assuming one nucleus per cell, and the average nuclear volume (Table I) were both increased similarly, to 1.4 times the control. This accounts for the observation that the relative volume of hepatocyte cytoplasm and nuclei were unchanged from controls (Table I). The increase in liver size, therefore, appeared to result from both hyperplasia and cellular hypertrophy.

After discontinuation of SaH 42-348 treatment, the number of total liver nuclei returned to control levels (Table I), as did the liver size. The reversal of hepatocyte size and number, however, was not accompanied by a reduction in the average nuclear volume (Table I). This was reflected in the increase in the relative nuclear volume after withdrawal of the drug (Table I).

Analysis of Ultrastructural Changes

The changes in hepatocyte organelles after treatment with and withdrawal from 0.10% SaH 42-348 have been summarized schematically in Figs. 1 and 2. As the liver size was in a dynamic state during the course of the experiment (Fig. 1), attention has been drawn to both the total liver and concentration values, the latter being expressed as the morphometric dimensions originally calculated for relative volume (cm^3/cm^3 hepatocyte cytoplasm) and surface density (m^2/cm^3 hepatocyte cytoplasm).

As is evident from Figs. 1 and 2, the most marked change in the relative content of hepato-

TABLE I

Effect of 0.10% SaH 42-348 in the Diet on the Relative Liver Weight, and the Relative Volume of Hepatocyte Cytoplasm and Hepatocyte Nuclei in Male Rats

Treatment	Relative liver wt	Hepatocyte cytoplasm	Hepatocyte nuclei			
		Vv	Vv		Total liver	Nuclear volume
	g/100 g rat	*cm^3/cm^3 liver*	*cm^3/cm^3 liver*	*(no./cm^3 liver) × 10^6*	*(no./100 g rat) × 10^6*	*$cm^3 \times 10^{-10}$*
Control diet	2.92 ± 0.15*	0.748 ± 0.011	0.092 ± 0.004	70.28 ± 3.79	212.1 ± 12.6	13.60 ± 0.82
0.10% SaH 42-348 21 days	6.04 ± 0.16‡	0.770 ± 0.013	0.090 ± 0.007	49.86 ± 2.35‡	301.2 ± 14.6‡	18.55 ± 1.55§
0.10% SaH 42-348 21 days, withdrawn 14 days	2.96 ± 0.03	0.717 ± 0.010	0.117 ± 0.006§	64.18 ± 4.36	191.2 ± 12.4	19.45 ± 1.32‡

* Values expressed as mean ± SE.
‡ Significantly different from control, $P < 0.001$.
§ Significantly different from control, $P < 0.01$.

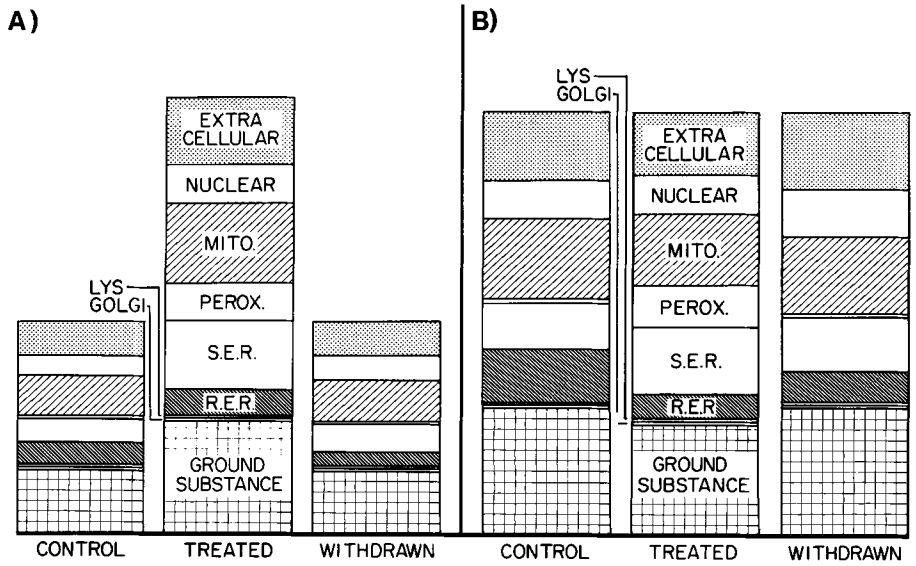

FIGURE 2 Schematic representation of volume changes in F-344 rat liver after treatment with and withdrawal from 0.10% SaH 42-348 in the diet. Expressed as (*a*) total liver values and (*b*) percent of liver.

cytes was a dramatic increase in surface area and volume of peroxisomes. During the 3-wk period of treatment, the relative volume of peroxisomes increased from the control value of 0.0146 to 0.1227 (cm^3/cm^3 hepatocyte cytoplasm). The increases in both relative volume and surface density were first observed on the 3rd day of treatment. These values increased steadily thereafter (Fig. 3). Upon withdrawal of the drug, the peroxisomal values decreased rapidly, returning to control levels within 8 days.

The increase in the collective peroxisomal volume was accompanied by an increase in the number of peroxisomes (Fig. 4*a*). However, while the relative volume of peroxisomes as a whole increased 8.4-fold, only a 3.1-fold increase occurred in the number of peroxisomes. A 2.5-fold increase in the average peroxisomal volume (Fig. 4*b*) accounted for the remaining change in the collective volume. The alteration in peroxisome size was further analyzed by direct measurement of peroxisome radii from the electron micrographs. Mitochondrial radii were also measured for comparative purposes. By use of this technique, the control radii of peroxisomes and mitochondria were found to be 0.299 ± 0.005 μm and 0.536 ± 0.010 μm, respectively (Fig. 5). The radii of these organelles in animals treated with 0.10% SaH 42-348 for 21 days were 0.362 ± 0.008 μm for peroxisomes and 0.534 ± 0.009 μm for mitochondria. The increase in the average peroxisome radius was statistically significant ($P < 0.001$).

The average radius of peroxisomes was also calculated from morphometric data by using the relationship between the relative volume and surface density, or by using the calculated average volume of peroxisome. Radii of 0.389 and 0.461 μm were obtained for control and treated animals, respectively, from the surface area-to-volume ratios, while radii of 0.332 and 0.451 μm for control and treated animals were derived from the average peroxisome volumes. Although the relative increase in size was similar for all three methods, higher values were obtained by using the latter two indirect methods. The experimental error between the surface area to volume ratio method and the direct measurement was 13.1% for control and 12.2% for treated groups. The errors between the average volume method and direct measurement were 5.2% and 11.1%, respectively. The average mitochondrial radii calculated from the volume to surface area relationship were also higher than the measured radii of 0.621 μm for control and 0.592 μm for treated animals, with experimental error of 7.4% and 5.2%, respectively.

On analysis of the lobular distribution of peroxisomes, the increases in peroxisomal relative volume and in surface area were found to be more marked in the centrilobular region (Fig. 6). A

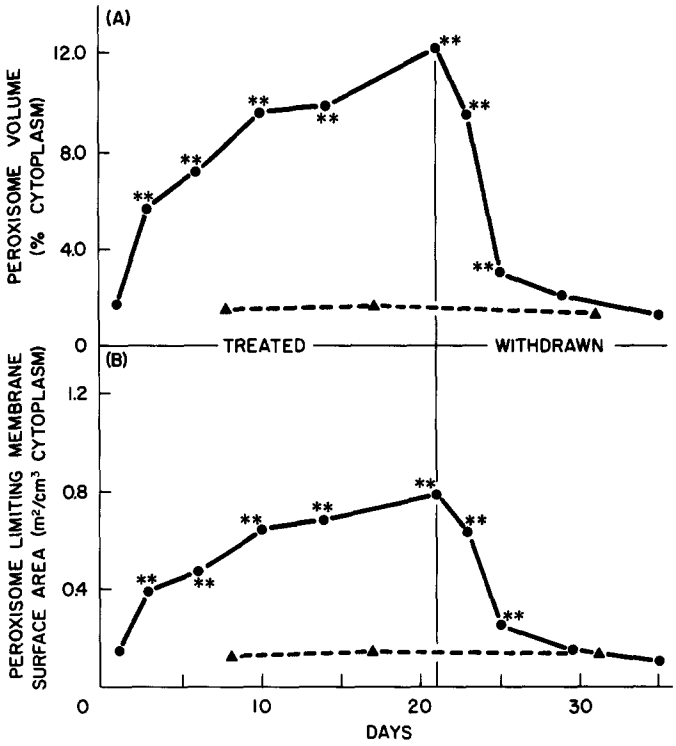

FIGURE 3 Morphometric values of hepatocyte peroxisomes in male F-344 rats receiving 0.10% SaH 42-348 in the diet (●——●) and control animals (▲—▲). (*a*) relative volume and (*b*) surface density of limiting membrane. Significant difference from pooled control values (** $P < 0.001$).

similar, but not as significant distribution was also observed in control animals.

Along with the peroxisomes, the volume and surface area of the smooth endoplasmic reticulum (SER) were increased to a greater extent than the change in liver size (Fig. 1). The relative volume and surface density were increased 0.5- and 1.0-fold, respectively, after 3 wk of treatment. The increase in SER values was observed on the 1st day of treatment and preceded the increase in peroxisomes. This was associated with a decrease in the total and relative values of rough endoplasmic reticulum (RER) on day 1 (Fig. 1). Thereafter, the total RER values remained above control values, but not to the extent of change in liver size, so that a significant decrease was found in the relative volume and surface density during treatment. During withdrawal, the SER values returned at first towards the control level, but after 4 days only a slight decrease occurred. The relative volume and surface density remained significantly above the control values after 14 days of withdrawal. As with peroxisomes, the values of SER volume and surface area for treated and control animals were highest in the centrilobular and lowest in the periportal regions (Fig. 6). No significant difference was noted in the lobular distribution of the RER.

The changes in mitochondrial volume and surface area paralleled the change in liver size (Fig. 1). The mitochondrial relative volume (Fig. 2) and surface densities of the outer mitochondrial membrane and inner membrane and cristae remained essentially unchanged during treatment and withdrawal periods. All three mitochondrial values were higher in periportal and lower in centrilobular regions, in contrast to peroxisomes and SER. The values for Golgi complexes and lysosomes were not altered significantly during treatment with 0.10% SaH 42-348. However, a significant increase in the relative volume and surface density of the lysosomes was noted on the 4th day of withdrawal (Fig. 7). No significant differences were encountered in the lobular distribution of these two organelles in either treated or control animals.

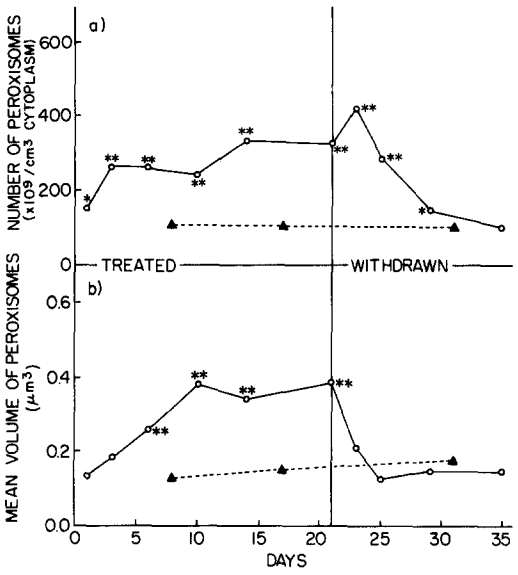

FIGURE 4 Morphometric values of hepatocyte peroxisomes in male F-344 rats receiving 0.10% SaH 42-348 in the diet (○——○) and control animals (▲---▲), (*a*) number of peroxisomes and (*b*) average volume of peroxisomes (** $P < 0.001$, * $P < 0.01$).

Peroxisome-Associated Enzymes

The activities of two enzymes associated with peroxisomes, catalase and carnitine acetyltransferase (CAT), and one mitochondrial enzyme, succinate dehydrogenase, were measured during the time course of the experiment. The total activities of catalase and CAT were found to increase rapidly during treatment, reaching maximum values by 10 days (Fig. 8). After withdrawal of the drug, the activities of these two enzymes decreased rapidly and reached control levels within 8 days. As illustrated in Fig. 8, the proportion of catalase activity in the supernatant fraction, as opposed to the particulate fraction, increased progressively from approximately one-half of the total in control animals to two-thirds in treated. After cessation of drug treatment, the proportion of supernatant activity returned to control levels. The supernatant activity of CAT was only one-fourth of the total activity in treated animals. The activity of succinate dehydrogenase, however, remained essentially unchanged during the entire time course. The subcellular distribution of the enzymes in control and 21 day-treated animals is presented in Fig. 9. Control catalase activity was found predominantly in the supernatant and mitochondrial peroxisome fractions. In treated animals, the catalase activity in supernate increased to a greater extent when compared to the mitochondrial + peroxisome fraction.

In control animals, the distribution of CAT activity appeared to be intermediate between those of catalase and succinate dehydrogenase except for a significant amount in the microsomal fraction. In contrast, the distribution of CAT in 21 day-treated animals was almost identical to that of catalase, suggesting that a large proportion of the increase in CAT activity occurred in the peroxisomes.

DISCUSSION

Liver Growth and Organelle Induction

It has been shown that the hepatomegaly induced in rats by treatment with SaH 42-348 arose from a combination of hypertrophy and hyperplasia of hepatocytes. The increase in hepatocyte size was associated with an elevated volume of peroxisomes and SER. The increase in peroxisome volume itself was due to an increase in both the size and the number of these organelles.

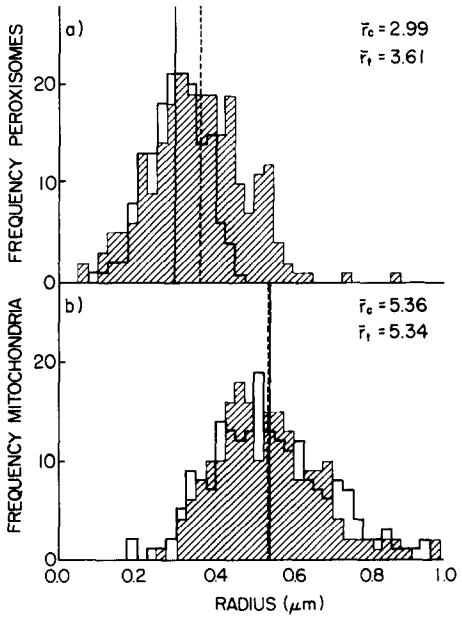

FIGURE 5 Histogram presenting profile radii of (*a*) peroxisomes and (*b*) mitochondria in control and 21 day-treated (cross-hatched) animals. Radii were estimated directly from photographs and taken as the geometric mean of the longest and shortest axis of transection. Approximately 200 transections measured for each group.

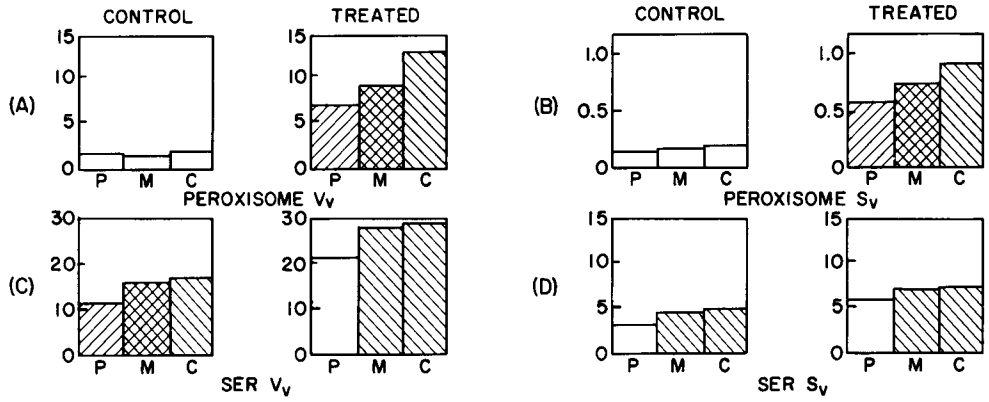

FIGURE 6 Morphometric analysis of lobular distribution of hepatocyte organelles in control male F-344 rats and animals treated with 0.10% SaH 42-348 in the diet for 21 days. Bars with no cross-hatching or cross-hatching in the same direction are not significantly different ($P < 0.01$). *P*, periportal; *M*, midzonal; *C*, centrilobular. (Lobular areas are defined in Materials and Methods.)

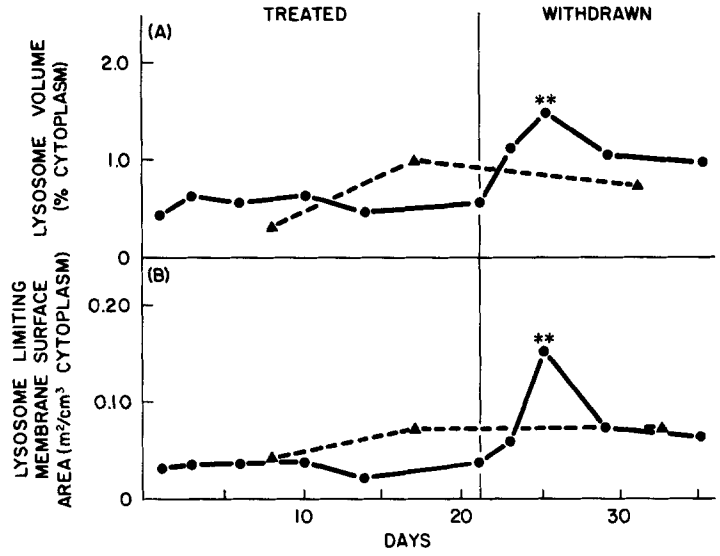

FIGURE 7 Morphometric values in male F-344 rats receiving 0.10% SaH 42-348 in the diet (●—●) and control animals (▲—▲). (*a*) Lysosome relative volumes, (*b*) lysosome surface density (** $P < 0.001$).

The increase in peroxisome size was demonstrated by direct measurements of profile radii and indirectly from morphometric data. The absolute values derived from these methods, however, did not agree for both peroxisomes and mitochondria. Similar discrepancies for mitochondrial measurements were noted in the results of other workers. Baudhuin and Berthet (2) measured mitochondrial radii directly from electron micrographs of subcellular fractions and obtained an average value of 0.382 μm, whereas calculation of the average radius, from the surface area to volume ratio reported, yielded a value of 0.437 μm. The experimental error between these two sets of values was 5.9%, as compared to 7.4 and 5.2%, respectively, in our measurements on mitochondria of control and treated animals. Calculation of the average mitochondrial radius from the data of Weibel and co-workers (48) on relative volume and surface area, and average mitochondrial volume, gave values of 0.372 μm and 0.552 μm, respectively. Accordingly, the indirect methods of measuring organelle radii do not appear to be representative. However, these methods appear

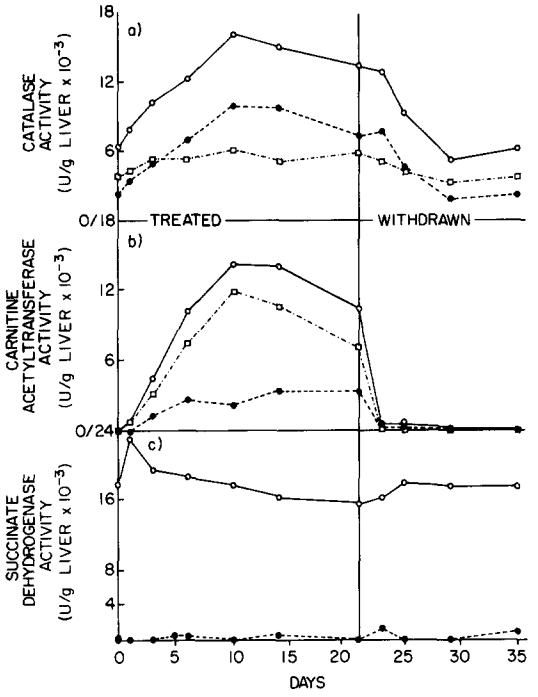

FIGURE 8 The time course of total (○——○), particulate (□---□), and supernatant (●—●) activities of hepatic (*a*) catalase, (*b*) carnitine acetyltransferase, and (*c*) succinate dehydrogenase during treatment with 0.10% SaH 42-348. Particulate activity represents the sum of the nuclear, mitochondrial-peroxisomal, and microsomal fractions.

adequate for comparison between two experimental groups, since relative changes did correspond with the direct measurements.

The proliferation of peroxisomes in these studies was accompanied by a proliferation of SER. An increase in hepatocyte SER has also been noted in animals treated with all other compounds capable of inducing peroxisome proliferation (17, 18, 20, 34–36). The proliferation of SER by these drugs may have been independent of the peroxisome proliferation and related to the induction of drug-metabolizing enzymes, as proposed for the compounds (such as phenobarbital [6]) which induce solely the SER. Slight increases in cytochrome P-450, metabolism of testosterone, and *N*-demethylase activity have been reported in rats treated with SaH 42-348 and clofibrate, suggesting induction of microsomal enzymes (40, 45). However, the increases were not as marked as those seen in animals treated with phenobarbital.

It is also possible that the increase in SER may have arisen in relation to the peroxisome proliferation. The composition of SER and peroxisome-limiting membranes has been shown to be similar (10). On ultrastructural observation, continuities have been noted frequently between peroxisomes and the SER (29). Therefore, it has been proposed that peroxisomes arise from the ER and may exist as a common pool in continuity with parts of the ER (30, 33). As the proliferation of SER preceded the increase in peroxisomes, SER proliferation in this instance may have occurred, in part, as a precursor to peroxisome proliferation. The finding of an initial decrease in the total amount of RER coincided with the initial increase in SER. It has been shown recently that the ratio of free to bound ribonucleoprotein increases in rats treated with clofibrate (31), an analogue of SaH 42-348, and may represent fall-off of polysomes from the RER, thus accounting in part for the initial changes in SER and RER values.

The possible relationship between the SER and peroxisomes was also exemplified by the similarity of their lobular distributions. The finding of a

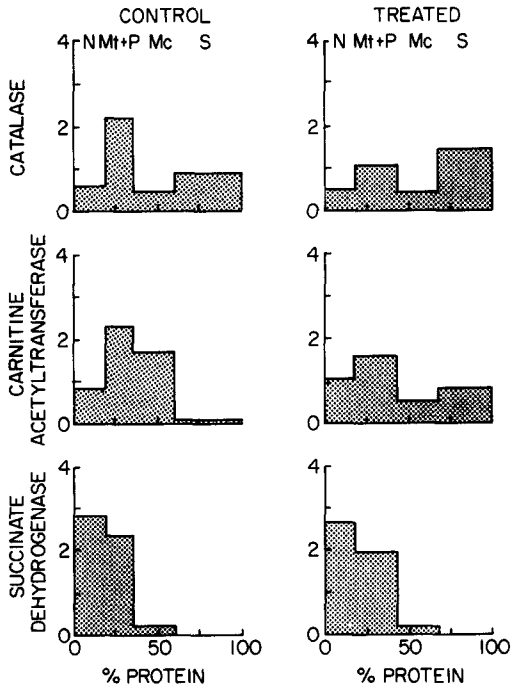

FIGURE 9 Subcellular distribution of (*a*) catalase, (*b*) carnitine acetyltransferase, and (*c*) succinate dehydrogenase. Ordinate expressed as cumulative percent of protein, abscissa as the relative specific enzyme activity (percent of total enzyme activity/fraction, divided by percent of total protein (fraction).

predominance of peroxisomes and SER in the centrilobular region is in agreement with Loud's morphometric studies (23) and with the findings of Wanson and co-workers that centrilobular hepatocytes separated on a Ficoll gradient contain more SER than the other cells (46).

Distribution patterns for the peroxisomes and SER were amplified, but remained unchanged after treatment with 0.10% of SaH 42-348. While this finding was not adequate to determine whether or not the proliferation of these organelles was initiated in a particular region, studies on clofibrate and nafenopin have shown (3) that cellular hypertrophy and, one may therefore, assume, the organelle proliferation were first noted in the centrilobular region. It therefore appears that, as with agents such as phenobarbital (41) and CCl_4 (32) which affect the SER, the initial effect of peroxisome proliferator drugs occurs in that region where these organelles predominate.

The relative volume and surface density of mitochondria were not affected by the administration of SaH 42-348. The total liver values of mitochondria increased in parallel with liver size, as reported previously for clofibrate and methyl clofenapate (28). Gear reported similar findings in rats treated with clofibrate using a particle counter on isolated liver mitochondria (14). Others, however, have noted an increase in the concentration of mitochondrial protein after treatment with clofibrate or methyl clofenapate (19). An increase in the protein content of an organelle may not necessarily contradict the lack of increase in the volume or surface area of that organelle. The increase in mitochondrial protein content may be due to an increase in the protein density within the matrix or membrane. However, in the case of drugs which induce proliferation of peroxisomes that sediment close to mitochondria, contamination of the mitochondrial fraction with peroxisomes may account for some of the changes observed in that fraction. The increase in total liver mitochondrial values in animals treated with this peroxisome proliferator is in contrast to the finding, that SER proliferation predominates and the total liver mitochondrial volume remains unchanged in animals treated with phenobarbital (43). Therefore, it appears that the increase in mitochondrial values is not a general phenomenon related to the liver growth.

Withdrawal

The liver size of treated rats reverted to control levels within 2 wk after the withdrawal of SaH 42-348 from the diet. This was accompanied by a similar reversal in all the organelles analyzed, except for the SER. In this and the previous study, which utilized clofibrate and methyl clofenapate (28), the values for the SER decreased, but only partially, so that they were still significantly elevated at 2 wk after withdrawal.

Bolender and Weibel have shown in their morphometric study that the SER induced by phenobarbital reverted to control levels within 5 days after withdrawal of treatment (4). It is conceivable that the prolonged period of regression for the SER in the present study may have arisen from the longer period of treatment, 21 days as opposed to 5 days in Bolender and Weibel's study. However, the increase in SER was of a similar magnitude at the end point of both experiments. Although the half-life of SaH 42-348 has not been reported, the rapid reversal in other parameters measured suggests that it is not too prolonged and may be comparable to that of phenobarbital. Therefore, if the mechanism of reversion in both cases was the same, one would expect to find a similar time period for the removal of these membranes.

Bolender and Weibel found that the removal of SER was associated with a marked increase in autophagic vacuoles (4). Therefore, the membrane removal was attributed to intracellular sequestration, followed either by secretion or by digestion through the lysosomes (7). In this study, when lysosomes were loosely defined to include autophagic vacuoles and dense bodies, a marked increase in the lysosome relative volume and in the surface density were seen on the 4th day after withdrawal of SaH 42-348 from the diet. A major decrease in the peroxisomes and SER did occur at the same time. Therefore, the lysosome system may have played an important role in the removal of these organelles, although direct evidence of increased numbers of autophagic vacuoles containing SER or peroxisomes was not encountered. If autophagy indeed played a role in the disposal of peroxisomes, the absence of autophagic vacuoles containing peroxisomes during reversal may be due either to rapid dissolution of the peroxisomal matrix upon ingestion or to incorporation of peroxisomes into the autophagic vacuole by fusion (8).

The turnover of peroxisomes in the natural state is not fully understood as yet, but a half-life of 1.5–3 days has been reported. The rate of regression in peroxisomes after the withdrawal of SaH

42-348 was compatible with this half-life. Therefore, the removal of peroxisomes may have resulted from cessation of induced synthesis and return to a normal steady state. When one considers the prolonged increase of part of the SER, peroxisomes may be removed by absorption of their contents within the SER (34) while, concurrently, portions of the SER are being sequestered. This would be compatible with the idea suggested earlier that peroxisomes may exist as a continuity with the SER (34). The persistent elevation of SER during reversal as shown by these morphometric studies is a strong indication that peroxisomal matrix proteins are removed by absorption and the empty limiting membranes contribute to increases in SER values.

Peroxisome-Associated Enzymes

The induction of peroxisomes was accompanied by an increase in the activity of two enzymes which are associated with peroxisomes. In all of the cases where these enzymes were studied, the activities of catalase and CAT have been found to increase after administration of drugs which induce peroxisome proliferation (27, 36, 37). The function of these enzymes in the induced peroxisomes has not yet been determined.

The distribution pattern of CAT in treated animals was almost identical to that of catalase when compared, after differential centrifugation, to the distribution of mitochondrial enzyme dehydrogenase and the peroxisomal enzyme catalase. The increased catalase and CAT activities in the supernatant fraction in treated animals appear to be due to release of these enzymes from peroxisomes during homogenization, since morphological evidence indicates that peroxisomal proteins accumulate in dilated SER channels and that these organelles are continuous with one another (22, 33). The distribution of hepatic CAT activity in rats treated with clofibrate was studied recently by Goldenberg et al. (16). They found approximately equal increases in the mitochondrial and peroxisomal fractions isolated by isopycnic gradient centrifugation. Our results appear to indicate a similar response in rats treated with SaH 42-348, an analogue of clofibrate, and the enzyme activity correlates well with the morphometric data on the increase in peroxisomes.

The function of CAT in peroxisomes, as well as in other organelles, has not been fully elucidated. The enzyme does appear to function in the transfer across the membranes of short-chain acyl groups and may play a role in biological acetylation and fatty acid synthesis (5, 13, 42). It therefore appears significant that such a marked increase in CAT activity occurs in association with peroxisome proliferation, since all compounds capable of inducing peroxisome proliferation also possess hypolipidemic activity (36). The association between CAT increase and peroxisome proliferation may in part help to clarify the relationship between peroxisomes and lipid metabolism.

The authors wish to thank Dr. Alex B. Novikoff for the helpful suggestions on morphometric analysis at the Histochemical Society Meetings held in April 1975 at Atlantic City, N.J. We thank Charles Sittler and Faye Brady for the excellent technical assistance and Ella Olson for typing the manuscript. SaH 42-348 was generously supplied by Sandoz Pharmaceuticals, Hanover, N.J.

This study was supported in part by United States Public Health Service Grants GM-23750 and GM-15956 and training grant GM-O-1783.

Received for publication 23 February 1976, and in revised form 19 July 1976.

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