NAFENOPIN-INDUCED HEPATIC MICROBODY (PEROXISOME) PROLIFERATION AND CATALASE SYNTHESIS IN RATS AND MICE

Absence of Sex Difference in Response

JARNARDAN K. REDDY, DANIEL L. AZARNOFF,
DONALD J. SVOBODA, and JADA D. PRASAD

From the Department of Pathology and Oncology and the Clinical Pharmacology and Toxicology Center, University of Kansas Medical Center, Kansas City, Kansas 66103

ABSTRACT

Nafenopin (2-methyl-2[p-(1,2,3,4-tetrahydro-1-naphthyl)phenoxy]-propionic acid; Su-13437), a potent hypolipidemic compound, was administered in varying concentrations in ground Purina Chow to male and female rats, wild type (Cs^a strain) mice and acatalasemic (Cs^b strain) mice to determine the hepatic microbody proliferative and catalase-inducing effects. In all groups of animals, administration of nafenopin at dietary levels of 0.125% and 0.25% produced a significant and sustained increase in the number of peroxisomes. The hepatic microbody proliferation in both male and female rats and wild type Cs^a strain mice treated with nafenopin was of the same magnitude and was associated with a two-fold increase in catalase activity and in the concentration of catalase protein. The increase in microbody population in acatalasemic mice, although not accompanied by increase in catalase activity, was associated with a twofold increase in the amount of catalase protein. The absence of sex difference in microbody proliferative response in nafenopin-treated rats and wild type mice is of particular significance, since ethyl-α-p-chlorophenoxyisobutyrate (CPIB)-induced microbody proliferation and increase in catalase activity occurred only in males. Nafenopin can, therefore, be used as an inducer of microbody proliferation and of catalase synthesis in both sexes of rats and mice. The serum glycerol-glycerides were markedly lowered in all the animals given nafenopin, which paralleled the increase in liver catalase. All the above effects of nafenopin were fully reversed when the drug was withdrawn from the diet of male rats. During reversal, several microbody nucleoids were seen free in the hyaloplasm or in the dilated endoplasmic reticulum channels resulting from a rapid reduction in microbody matrix proteins after the withdrawal of nafenopin from the diet. Because of microbody proliferation and catalase induction with increasing number of hypolipidemic compounds, additional studies are necessary to determine the interrelationships of microbody proliferation, catalase induction, and hypolipidemia.

INTRODUCTION

Microbodies (peroxisomes), the cytoplasmic constituents described originally in kidney (1) and liver (2), have only recently been recognized as ubiquitous structures in animal (3–5) and plant

cells (6). de Duve and associates (7–8) investigated the biochemical composition of microbodies of rat liver and showed that these structures possess catalase and several oxidative enzymes. It is evident from several studies that catalase is the only constant component of peroxisomes derived from different sources, whereas the hydrogen peroxide-generating oxidative enzymes that are associated with catalase differ in number and in specificity (9). On the basis of the cytochemical localization of catalase with the alkaline 3',3'-diaminobenzidine medium (10, 11), microbodies have been identified in tissues other than liver and kidney. Additional studies, however, are necessary to characterize the composition of these catalase-containing structures in tissues other than liver and kidney either by biochemical or cytochemical procedures; and if the structures are found to possess at least one hydrogen peroxide-producing oxidase, they can then be unequivocally regarded as peroxisomes.

Although considerable progress has been made in the understanding of the functional role of peroxisomes in plant cells, the importance of these organelles in cellular metabolism in animal tissues remains to be elucidated. However, several studies suggest that microbodies may play a significant but as yet unexplained role in a variety of functions such as in gluconeogenesis, in cholesterol and steroid metabolism, in the detoxification of hydrogen peroxide, etc. (7, 12, 13). Studies with ethyl-α-p-chlorophenoxyisobutyrate (CPIB) have clearly established that this compound is valuable in investigating the biological behavior of microbodies under different experimental situations and also suggested a possible relationship between microbodies and lipid metabolism (13–18). CPIB is a potent hypolipidemic drug which lowers serum triglycerides and cholesterol in man (19) and in experimental animals (20). Administration of this drug to male rats results in a significant increase in the number of microbody profiles in liver cells (14–16). The CPIB-induced microbody proliferation in male rat liver was associated with a marked increase in catalase activity resulting from enhanced rate of synthesis of this enzyme (21). However, when CPIB was administered to female rats in doses identical to those given to male rats for the same duration, there was no appreciable increase either in the number of microbody profiles or in the liver catalase activity. Similar sex differences in microbody proliferative and catalase response were observed in C_3H mice and in wild type (Cs^a strain) mice given CPIB,

whereas no sex difference in microbody proliferative response was noted in acatalasemic (Cs^b strain) mice (17).

Recently we have demonstrated that administration of nafenopin (2-methyl-2[p-(1,2,3,4-tetrahydro-1-nephthyl)phenoxy]-propionic acid; Su-13437) to male rats, by gavage at 100 mg/kg body weight daily for 1–2 wk, results in a marked proliferation of hepatic microbody profiles with simultaneous increase in catalase activity (22). Nafenopin is structurally related to CPIB and on an equivalent weight basis is approximately five times as potent as CPIB in producing hypolipidemia and hepatomegaly (23, 24). In the present investigation varying concentrations of nafenopin were administered in ground Purina Chow ad lib to both male and female rats, wild type (Cs^a strain) mice and acatalasemic (Cs^b strain) mice, in order to determine the dose-response relationship and to ascertain the species and sex differences, if any, in microbody proliferative response and inducibility of liver catalase. The results indicate an absence of sex difference in microbody proliferative response in nafenopin-treated rats and wild mice, whereas CPIB-induced microbody proliferation occurred only in males (16). Accordingly, nafenopin can be used as an inducer of microbody proliferation and of catalase enzyme synthesis in both sexes of rats and mice. These studies also emphasize the significance of hypolipidemic drugs as effective tools for investigating the interrelationship of microbodies with lipid metabolism.

MATERIALS AND METHODS

Animals and Treatment

The inbred rats used in these experiments were of the F-344 strain (Simonson Laboratories Inc., Gilroy, Calif.) weighing between 125 and 175 g, which were maintained in individual cages. The wild type (Cs^a strain) mice and the acatalasemic (Cs^b strain) mice used in these experiments were 2–6 mo old and weighed between 20 and 30 g. Nafenopin (CIBA Pharmaceuticals, Summit, N. J.) was added to the ground Purina Chow in concentrations of 0.0125%, 0.05%, 0.125%, and 0.25% and was thoroughly mixed. Preliminary studies in rats indicated that nafenopin at 0.125% and 0.25% dietary levels was very effective in inducing microbody proliferation. Accordingly, studies in wild type and acatalasemic mice were conducted with these two dose levels. At the end of 1, 2, 3, 4, 6, and 8 wk, liver tissue for morphological studies was obtained from two to three animals from each of the treated groups after laparot-

omy or sacrifice. The *reversal* of nafenopin-induced microbody proliferation was investigated in male rats. After receiving 0.25% nafenopin for 6 wk, animals were killed on days 1, 3, 5, 7, 10, and 14 after withdrawal of the drug for ultrastructural studies and catalase assay.

Morphology

For light microscope examination, samples of liver were fixed in neutral buffered formaldehyde and embedded in paraffin. 3–5 μm sections were stained with hematoxylin and eosin. For electron microscope studies, tissues were fixed for 1–2 h at 4°C in 2% osmium tetroxide buffered with S-collidine to pH 7.4, and processed as described previously (15). For cyto-chemical localization of catalase, selected samples of liver from treated and untreated animals were fixed in 2.5% glutaraldehyde buffered with 0.1 M sodium cacodylate, pH 7.4, for 4 h at 4°C. After fixation, the tissues were rinsed overnight in 0.1 M cacodylate buffer containing 0.2 M sucrose. Then the tissues were finely chopped with a Sorvall "chopper" (Ivan Sorvall, Inc., Newtown, Conn.) and incubated at 37°C for 30–45 min in the 3′,3′-diaminobenzidine oxidation medium of Novikoff and Goldfischer (10) and processed for electron microscope examination.

Assay of Liver Catalase Activity

After determining that nafenopin at 0.125% and 0.25% caused maximal proliferation of microbodies, in both male and female animals, we limited the bio-chemical studies of catalase to these two dietary levels. The animals were killed by light ether anaesthesia and the livers were removed and weighed. 5% liver homogenates were prepared to which sodium deoxy-cholate (DOC) was added to give a final DOC concentration of 0.5% (25). This mixture was incubated at room temperature for 30 min and then centrifuged in a Spinco model L ultracentrifuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) using a 50 rotor at 35,000 rpm for 85 min. After centrifugation the lipid-containing layer at the top was carefully removed and the supernate was assayed for catalase activity at 25°C by the spectrophotometric method described by Lück (26). Total proteins were determined by the method of Lowry et al. (27).

Quantitation of Liver Catalase Protein

IN RATS: Content of catalase protein in normal and nafenopin-treated male and female rats was determined by immunotitration method described by Ganschow and Schimke (25).

IN WILD AND ACATALASEMIC MICE: In order to determine the content of liver catalase protein among control and nafenopin-treated wild type (Cs[a]

strain) and acatalasemic (Cs[b] strain) mice, immuno-precipitation of protein reactive with antibody specific for catalase was undertaken. The anticatalase serum was obtained by injecting rabbits with catalase puri-fied from the livers of Cs[a] strain mice. By the Ouch-terlony double diffusion method, this antiserum gave single precipitin lines with wild and acatalasemic liver extracts.

The content of liver catalase protein in control and nafenopin-treated wild type male and female mice was determined by the immunotitration method referred to above (25). Quantitation of liver catalase protein in acatalasemic mice by the immunotitration method could not be performed, because of low levels of catalase activity (17, 28) and also because of rapid inactivation of catalase when incubated at 37°C (29). In order to compare the amount of catalase protein in livers of acatalasemic and wild type mice, equal amounts of liver extracts from normal and nafenopin-treated animals were incubated with quantities of antiserum that were found to neutralize the catalase protein in normal and nafenopin-treated wild type mice. The immunoprecipitates were centrifuged and washed twice with cold 0.15 M sodium chloride. The washed precipitates were dissolved in 0.1 M sodium hydroxide and the absorbance at 280 nm was measured.

Serum Cholesterol and Triglycerides

All animals were lightly anesthetized with ether. Blood was collected from the inferior vena cava from nonfasted animals between 9 and 10 a.m. Serum total cholesterol and/or glyceride glycerol were determined after quantitative separation on silicic acid from phos-pholipids by the micromethod described by Azarnoff (30).

RESULTS

Studies in Rats

MICROBODY PROLIFERATION: With 0.0125% of nafenopin for 2 wk, the liver weights were only slightly increased. Nafenopin given at 0.125% and 0.25% produced a marked increase in liver weights in both male and female rats (Table I). Light microscope examination of these livers revealed greatly enlarged hepatocytes con-taining abundant acidophilic, finely granular cytoplasm. During the initial states of nafenopin treatment, numerous mitoses of liver cells were encountered. Autoradiographic studies, after [³H]thymidine pulse, during the first week of nafenopin administration showed numerous la-beled hepatocyte nuclei in male and female rats.[1]

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[1] Reddy, J., and Prasad, J. Unpublished observations.

TABLE I

Effect of Feeding F-344 Rats with Nafenopin on Liver Weight, Liver Catalase Activity, Serum Cholesterol, and Serum Glyceride-Glycerol Concentrations

Group*	No. of animals	Liver weight‡	Liver catalase activity‡	Serum cholesterol‡	Serum glyceride-glycerol‡
		g/100 g body weight	*U/mg protein*	*mg/100 ml*	*mg/100 ml*
Male rats					
Controls	6	3.75 ± 0.42	38.0 ± 3.9	82 ± 5	10.6 ± 2.8
Nafenopin (0.125%)	5	8.09 ± 0.52	74.9 ± 5.1	86 ± 9	2.9 ± 0.6
Nafenopin (0.25%)	6	8.34 ± 0.48	86.1 ± 4.3	79 ± 8	1.6 ± 0.2
Female rats					
Controls	6	3.21 ± 0.36	31.2 ± 3.7	76 ± 4	9.7 ± 2.4
Nafenopin (0.125%)	4	7.95 ± 0.18	71.4 ± 4.2	66 ± 4	2.5 ± 0.3
Nafenopin (0.25%)	5	8.10 ± 0.04	82.7 ± 1.25	74 ± 6	2.3 ± 0.5

* Nafenopin was administered in ground Purina Chow for 4 wk.
‡ Mean ± standard error.

Increase in the number of microbody profiles was evident in both sexes of rats given various dietary concentrations of nafenopin. 1-μm sections of Epon-embedded material clearly suggested that the increase in microbodies is generalized in the cytoplasm of liver cells throughout the entire liver lobule. With 0.0125% nafenopin, the microbody increase was slight when compared to the degree of proliferation encountered with higher dose levels. The microbody profiles were most numerous in liver cells of male and female rats (Fig. 1) given 0.125% nafenopin. With 0.25% nafenopin, there was no additional increase in number of microbody profiles. The microbody proliferation in both sexes of rats treated with 0.125% and 0.25% nafenopin was found to be of the same magnitude. Prolonged treatment with nafenopin resulted in the persistence of an increased number of peroxisomal profiles in male and female rats. Conspicuous variations in size and shape of microbody profiles were apparent. Nucleoids were observed in many microbody profiles of different sizes and configurations. Abnormalities in size and shape of peroxisomal profiles were striking in animals given 0.0125% nafenopin. Several of these microbody profiles contained striations in the matrix (Fig. 2), similar to those seen in CPIB-treated animals (14). A number of microbody profiles in liver cells of animals on prolonged nafenopin treatment possessed a somewhat flocculent matrix. However,

all these organelles stained positively for catalase when incubated in the alkaline 3',3'-diaminobenzidine medium. This reaction was inhibited when tissues were incubated in the presence of aminotriazole.

In addition to showing microbody proliferation, the liver cells of nafenopin-treated animals revealed marked proliferation of smooth endoplasmic reticulum. Isolated channels of rough endoplasmic reticulum were also frequently encountered. Mitochondria appeared normal in size, shape, and number. They did not show any inclusions or other obvious structural abnormality (Fig. 1). There was no increase in the number of lysosomes or lipid droplets in nafenopin-treated livers. Many hepatocyte nucleoli were hypertrophied.

MICROBODY REVERSAL: Rats treated with nafenopin for 6 wk showed increase in the number of microbody profiles to the same extent as evidenced at 4 and 8 wk. Gradual depletion in the number of microbody profiles was noted during the first 7 days after withdrawal of nafenopin. By 14 days after the drug was discontinued, the number of microbody profiles returned to normal. The reduction in microbody profiles was imperceptible in that there was no appreciable evidence of cellular autophagy. Detailed examination of livers during the first week of nafenopin withdrawal revealed certain interesting morphological features (Figs. 3–7). Marked decrease in electron
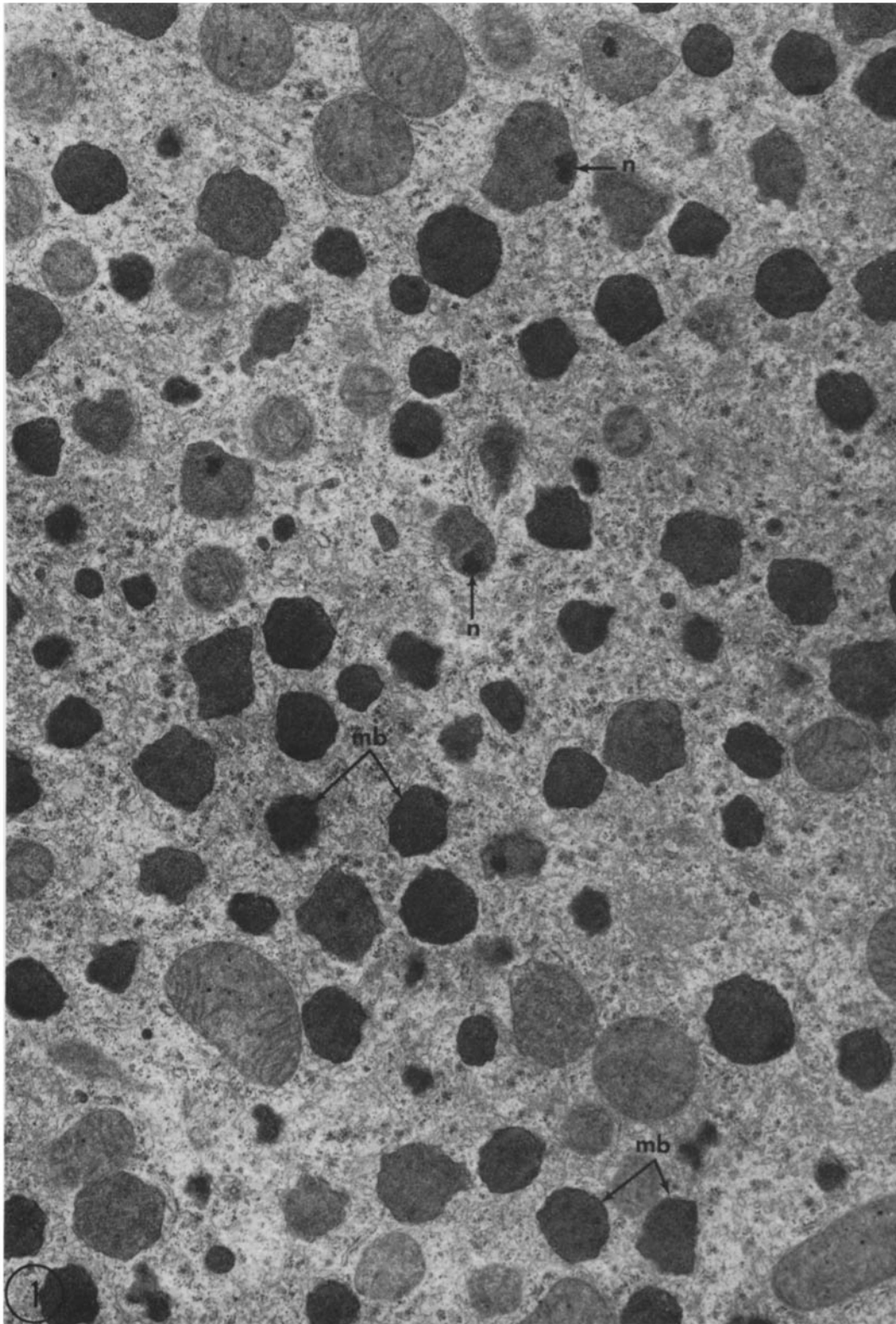
FIGURE 1 Female rat liver. Nafenopin 0.125% in the diet for 4 wk. The microbody proliferation in female rat liver cells was comparable to that observed in male rats. Significant variation in size and shape of microbody profiles (*mb*) is evident. Nucleoids (*n*). × 14,500.

opacity of the matrix was noted in some micro-body profiles. Several dilated endoplasmic reticulum channels contained crystalline microbody nucleoids or cores (Figs. 3 and 4) without the background matrix proteins, suggesting a gradual depletion of matrix proteins presumably due to cessation of synthesis after nafenopin withdrawal. Extrusion of nucleoids into hyaloplasm was also seen. At 7 days after withdrawal of nafenopin, occasional examples of nucleoids lying free in the hyaloplasm were also encountered (Figs. 6 and 7).

LIVER CATALASE: The results in Table I indicate that the activity of catalase in the liver in both male and female rats increased about twofold on administration of 0.125% or 0.25% nafenopin. When liver catalase activities were determined in male rats treated with 0.25% nafenopin for 2–14 days, it was found that the activity of catalase increases progressively, reaching the steady state level in 8–12 days. Nafenopin appeared as effective as CPIB in increasing the liver catalase activity. It is of interest to note that the induced steady state of catalase activity with these two drugs is nearly the same, i.e. approximately twice that of the normal steady state.

The results of immunotitration of liver catalase in male and female rats treated with 0.25% nafenopin for 4 wk are shown in Fig. 8. The amount of anticatalase serum needed to precipitate completely the catalase activity from 1 ml of 5% liver extract of either male or female rat treated with nafenopin was two times greater than that required for extracts from livers of untreated controls. These results indicate that the amount of catalase protein in liver extracts of male and female rats given nafenopin was the same and that nafenopin causes a twofold increase in the amount of liver catalase protein when compared to controls.

By 10 days after withdrawal of nafenopin from the diet, the catalase activity in liver reverted to normal values which paralleled the reduction in the number of hepatic microbody profiles.

SERUM CHOLESTEROL AND SERUM GLYC-ERIDE LEVELS: Administration of nafenopin at 0.125% and 0.25% to both male and female rats produced no significant cholesterol-lowering effect at 4 wk. In direct contrast, however, a profound reduction in serum glyceride levels was obtained in both males and females (Table I). Within 7–10 days after withdrawal of the drug the serum glyceride levels returned to normal,

which paralleled the reduction in catalase activity and the number of microbody profiles.

Studies in Mice

MICROBODY PROLIFERATION: Nafenopin was given to both sexes of wild type (Cs^a strain) mice and acatalasemic (Cs^b strain) mice at dietary levels of 0.125% and 0.25%. The gain in body weight was not affected at 0.125% dose level, whereas at 0.25% level there was some reduction in weight gain. However, at both these levels, nafenopin produced a marked increase in the weight of the liver in both males and females. In wild type mice fed 0.125% nafenopin for 4–8 wk the liver weight was approximately 25% of the body weight (Table II). Similar increases in liver weight were also seen in both sexes of acatalasemic mice treated with nafenopin. Examination of these livers with the light microscope showed large polygonal liver cells with abundant cytoplasm. Several mitotic figures were seen in the livers of animals killed during the first 10 days of nafenopin administration, suggesting that nafenopin-induced increases in liver weight are due in part to cell multiplication and cellular hypertrophy.

The microbody proliferation was most pronounced in the livers of both sexes of wild type and acatalasemic mice given 0.125% nafenopin (Fig. 9). Further increase in the number of microbody profiles was not seen with the higher dose level. In previous studies, we did not observe any increase in the number of microbody profiles in the female wild type (Cs^a strain) mice treated with CPIB, although CPIB produced a marked increase in hepatic microbody population in both sexes of acatalasemic (Cs^b strain) mice (17). The absence of sex difference in microbody proliferation in response to nafenopin, in rats and mice, is of particular interest.

The microbody profiles were numerous and showed a great deal of variation in size and shape. Continuities between adjacent microbody profiles and between microbody profiles and smooth endoplasmic reticulum channels were noted frequently (Figs. 10 and 11). Structural changes in mitochondria were not seen, and because of marked increase in microbody profiles, the mitochondria appeared sparse. Proliferation of smooth endoplasmic reticulum was observed in the liver cells of all animals treated with nafenopin.
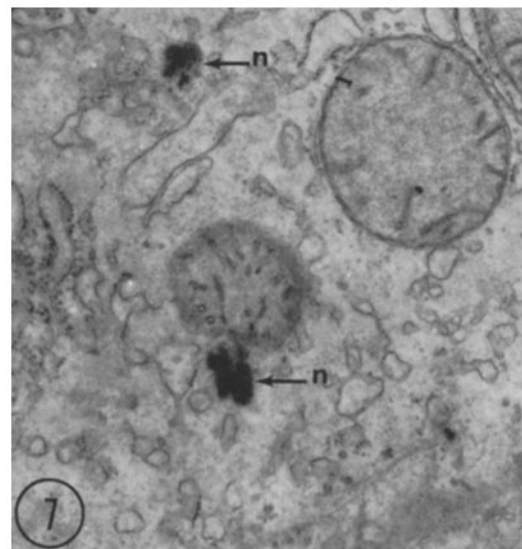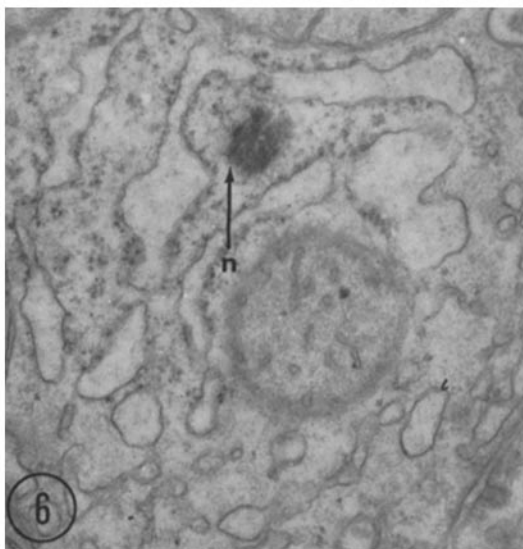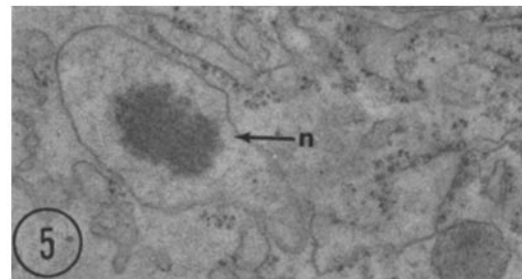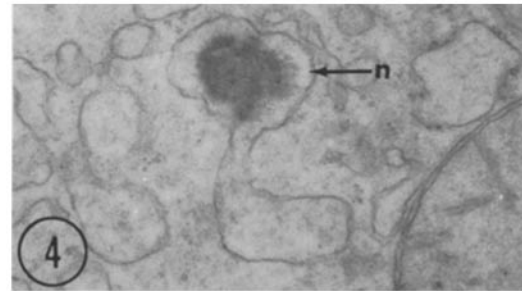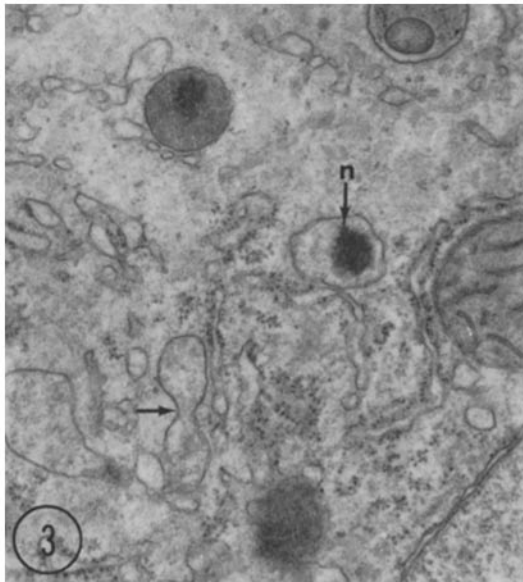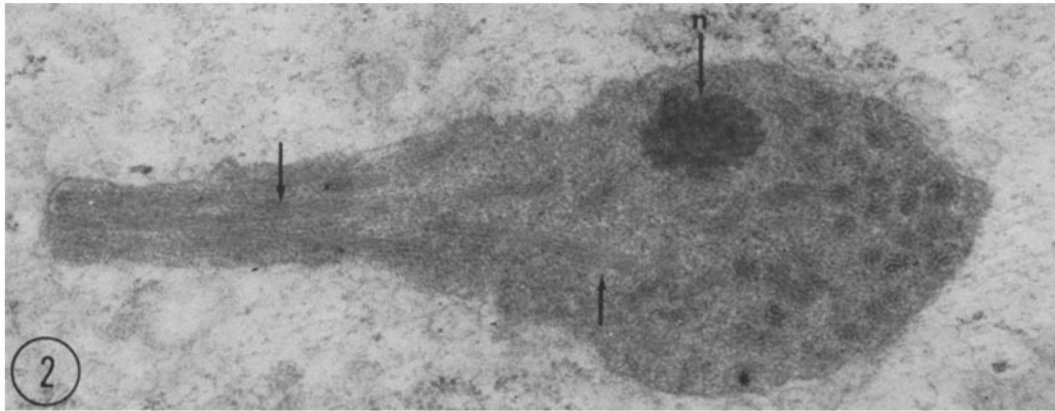
LIVER CATALASE: The results of the determination of catalase activity in livers of acatalasemic and wild type mice are shown in Table II. After nafenopin treatment, the catalase activity in male and female wild type mice (Cs^a strain) was significantly increased. The amount of catalase protein in liver of untreated and treated Cs^a mice was determined by the immunotitration method.
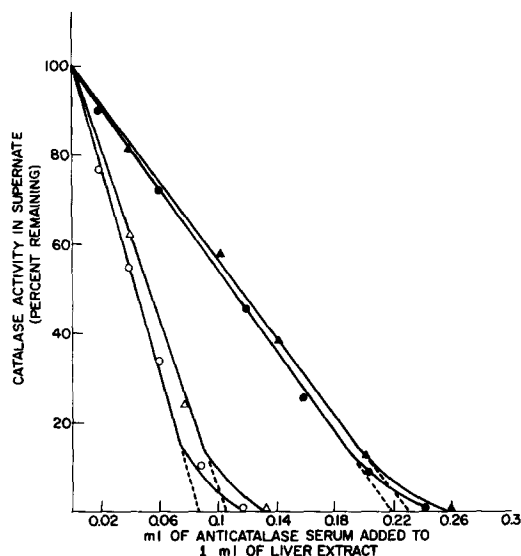
FIGURE 8 Quantitation of liver catalase by immunoprecipitation in male and female rats treated with 0.25% nafenopin for 4 wk. Increasing quantities of anticatalase serum were added to 1-ml samples of deoxycholate-treated liver extracts from normal males (△—△), normal females (○—○), nafenopin-treated males (▲—▲) and nafenopin-treated females (●—●). Dashed lines are extrapolations of the linear portions of the titration curves to zero catalase activity.

For complete precipitation of the catalase activity from 1 ml of 2.5% liver extract of Cs^a mice treated with nafenopin for 8 wk, approximately 0.64 ml of anticatalase serum was required, whereas only 0.31 ml antiserum was required to precipitate the catalase activity from 1 ml of 2.5% liver extracts from untreated wild mice. From these findings it can be reasonably assumed that a twofold increase in concentration of catalase protein occurs in livers of Cs^a mice treated with nafenopin.

Acatalasemic (Cs^b) mice treated with nafenopin showed only a minimal increase in liver catalase activity (Table II), although the microbody population in liver cells was comparable to that encountered in male and female wild type (Cs^a) mice. Studies of Aebi et al. (29) demonstrated that acatalasemic mutant mice have an unstable catalase that is unusually heat labile. The catalase in the present studies was assayed on liver extracts treated earlier with DOC and kept at room temperature for 30 min (see Materials and Methods). Accordingly, the low levels of catalase in nafenopin-treated Cs^b mice may be, in part, due to inactivation at room temperature.

To determine the quantity of catalase protein in acatalasemic mice, 0.31 ml and 0.64 ml of anticatalase serum were added, respectively, to 1 ml samples of 2.5% liver extracts from control and nafenopin-treated animals. The amount of anticatalase serum used was the same that was found to precipitate all the catalase activity in wild type mice by the immunotitration method (see above). The amount of protein precipitated from nafenopin-treated, acatalasemic mouse liver extracts was approximately twice that precipitated from control mouse liver extracts. Furthermore, the amounts of protein precipitated

FIGURE 2 Male rat treated with 0.0125% nafenopin for 2 wk. Several microbody profiles contained striations in the matrix (arrows). Nucleoid (n). × 65,000.

FIGURES 3–7 Figures 3–7 are from the livers of male rats killed between 2 and 10 days after withdrawal of nafenopin after they have been on the drug for 6 wk.

FIGURES 3–5 Microbody nucleoids (n) are seen within the irregular endoplasmic reticulum channels within 5–7 days after discontinuation of nafenopin. Electron-opaque microbody proteins are not seen in these membranes. In Fig. 3, the dumb-bell-shaped channel (arrow) contains a residue of electron-opaque material similar to that present in the channel containing nucleoid, possibly suggesting that the matrix proteins have dried up after withdrawal of nafenopin. Figs. 4 and 5. × 71,000. Fig. 3. × 49,000.

FIGURES 6 and 7 Nucleoids (n) are seen in the hyaloplasm. By 5–7 days after removal of nafenopin from the diet, some microbody nucleoids were seen lying free in cytoplasm after dissociating from microbody profiles. × 52,000.

TABLE II

Effect of Feeding Wild Type (Cs^a Strain) Mice and Acatalasemic (Cs^b Strain) Mice with Nafenopin on Liver Weight, Liver Catalase Activity, and Serum Glyceride-Glycerol Levels.

Group*	No. of animals	Liver weight	Liver catalase activity	Serum glyceride-glycerol
		g/100 g body weight	*U/mg protein*	*mg/100 ml*
Wild type (Cs^a) mice				
Males control	6	5.3 ± 0.30	38.8 ± 3.1	11.6 ± 1.8
Males nafenopin	4	24.6 ± 2.80	76.3 ± 5.6	4.2 ± 0.4
Females control	5	4.9 ± 0.26	37.2 ± 4.3	10.5 ± 1.3
Females nafenopin	4	26.5 ± 1.70	71 ± 5.4	4.1 ± 0.6
Acatalasemic (Cs^b) mice				
Males control	6	5.6 ± 0.25	10.5 ± 3.6	5.7 ± 1.7
Males nafenopin	4	18.7 ± 1.90	15.2 ± 2.7	1.9 ± 0.6
Females control	4	5.0 ± 0.40	11.3 ± 3.2	5.3 ± 2.2
Females nafenopin	4	19.8 ± 2.75	13.4 ± 2.8	2.1 ± 0.8

* Nafenopin (0.125%) was given in ground Purina Chow for 8 wk.

from the liver extracts of acatalasemic and wild type mice were also comparable. From these results it can be assumed that nafenopin causes approximately a twofold increase in the quantity of liver catalase protein in acatalasemic mice.

SERUM GLYCERIDE LEVELS: The results listed in Table II indicate that serum glyceride-glycerol levels are considerably lower in the acatalasemic mice than in the wild type mice. These findings in untreated controls are in agreement with the observations of Goldfischer et al. (31). After treatment with 0.125% nafenopin for 8 wk, the serum glyceride levels in both sexes of acatalasemic and wild type mice were markedly lowered (Table II). Serum cholesterol levels of these mice were not determined in this study.

DISCUSSION

Unlike other cellular constituents, microbodies (peroxisomes) in hepatic parenchymal cells are infrequently altered in number or morphology by experimental manipulations. However, markedly reduced activities of catalase and/or other peroxisomal enzymes in the liver have been observed in certain conditions, particularly in tumor-bearing animals (32). Quantitative studies to date indicate that CPIB and acetylsalicylic acid are the only known compounds that are capable of inducing significant increase in the number of microbody profiles in male rat liver cells and that the microbody proliferative response induced by CPIB is more pronounced in comparison to that induced by acetylsalicylic acid (14, 15, 33). Concomitant

with the increase in microbody profiles, the liver catalase activity is increased twofold after CPIB treatment due to enhanced synthesis of this enzyme (21). Additional studies by Svoboda, Azarnoff, and Reddy (16) demonstrated that in rats, CPIB-induced microbody proliferation and rise in catalase levels are dependent, to a significant degree, upon male sex hormones.

In the present study, microbody proliferation in both male and female rats treated with higher doses of nafenopin (0.125% and 0.25%) was very pronounced. Marked increase in the number of microbody profiles was also observed in both sexes of wild type and acatalasemic mice given nafenopin. The microbody proliferation induced with 0.125% nafenopin in these animals was of the same degree as that induced by 0.25% CPIB (14, 15, 17). In addition, these studies have also shown a twofold increase in the hepatic catalase activity and in the amount of catalase protein in both sexes of rats and wild type mice treated with nafenopin. Although the increase in catalase activity was not significant in acatalasemic mice given nafenopin, the amount of liver catalase protein in these animals was approximately equal to that found in nafenopin-treated wild type mice.

Nafenopin, like CPIB, is a phenolic ether which produces in rats a significant reduction in serum triglycerides, but does not significantly reduce the serum cholesterol (24). Serum glycerol-glycerides were significantly lowered in rats and mice given nafenopin in the present investigation. The studies of Best and Duncan (24) indicate that somewhat
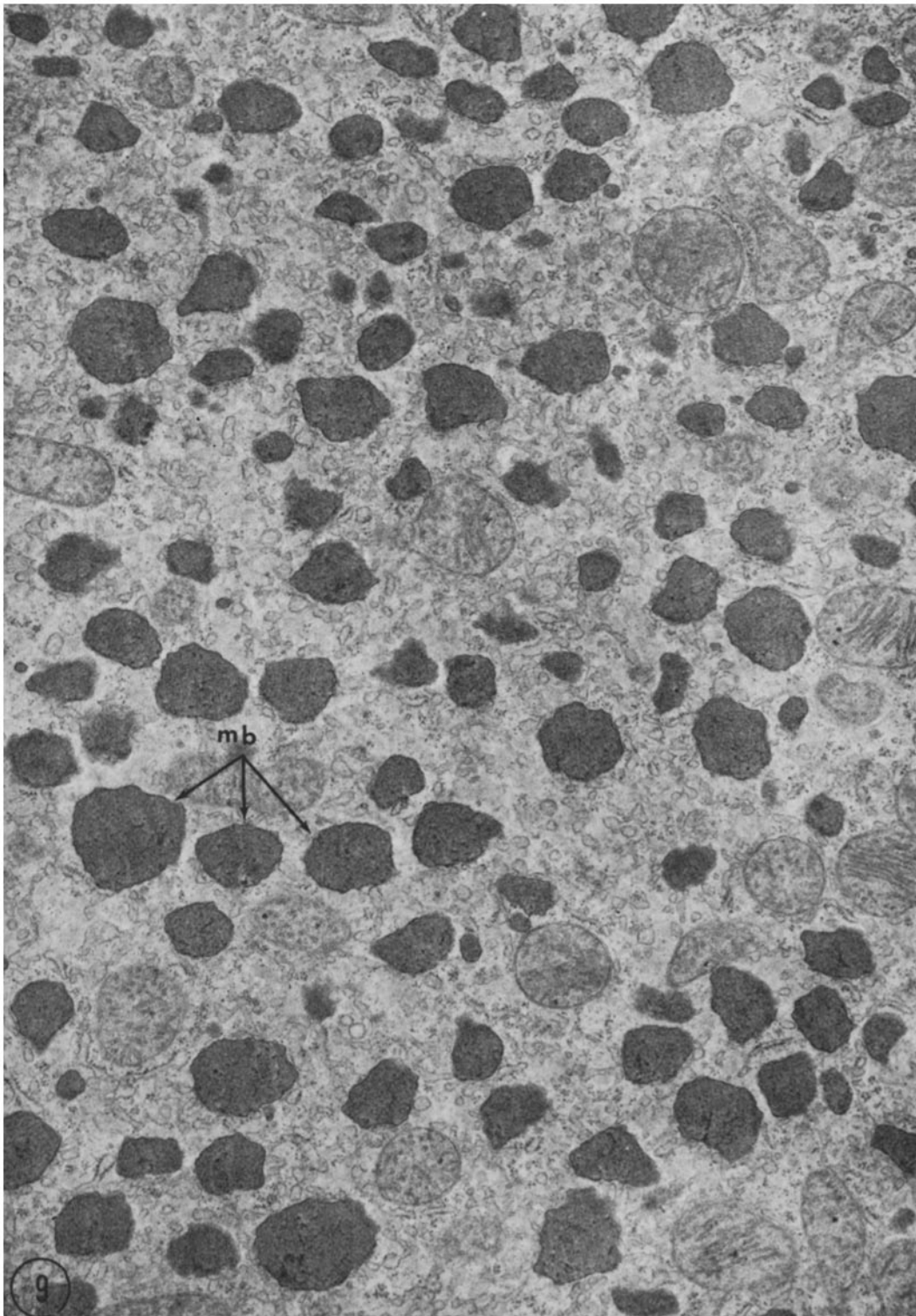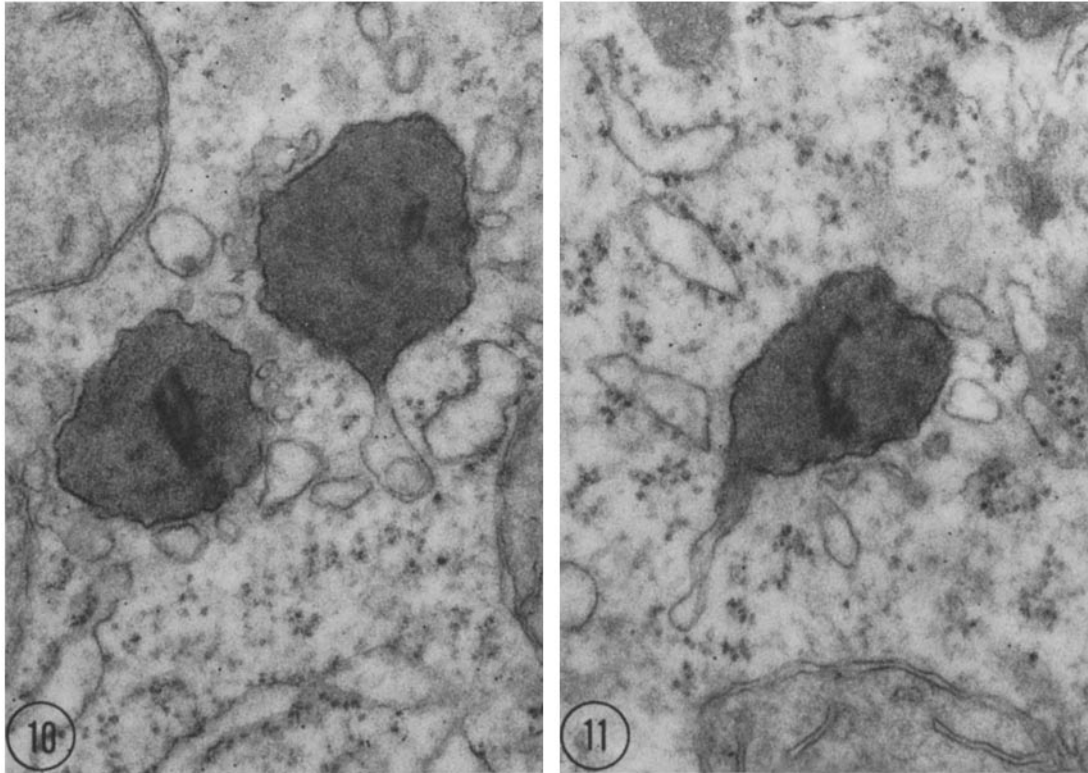
FIGURE 9 Female wild type mouse (Cs^a strain). Nafenopin 0.125% for 4 wk. Marked increase in number of microbody profiles (mb) is seen. The microbody proliferation in male Cs^a mice treated with nafenopin was of similar magnitude. × 19,000.

Figures 10 and 11 Wild type male mouse. Nafenopin 0.125%, 8 wk. Microbody profiles (*mb*) containing nucleoids are continuous with the smooth endoplasmic reticulum channels (arrows). × 54,000.

less than one-fifth as much nafenopin as CPIB is required to produce similar degrees of hepatomegaly and reduction in serum triglycerides in rats. With 0.125% and 0.25% nafenopin, the liver weights of rats in the present study were significantly higher than in rats given 0.25% CPIB (15). The hepatomegaly was most striking in wild type and acatalasemic mice treated with 0.125% nafenopin. In these animals, approximately 3 wk of nafenopin administration resulted in massive enlargement of livers, which accounted for one-fifth to one-fourth of the total body weight. Prolonged treatment for 8 wk did not show significant additional increase in liver weight. Although microbody proliferation undoubtedly contributed to hepatocellular hypertrophy in these animals, preliminary studies using [³H]thymidine autoradiography suggest that nafenopin induces hepatocellular proliferation during the initial stages, resulting in an increase in liver cell population. After the regenerative phase, the liver cells appear to undergo marked hypertrophy (34).

Previous studies from this laboratory demon-

strated a distinct sex difference in microbody proliferative response and in liver catalase inducibility in rats, C₃H mice, and in wild type (Cs^a) mice, after CPIB administration (15–17). In females, CPIB failed to induce microbody proliferation and increase in liver catalase activity. However, when castrated female rats were given testosterone propionate together with CPIB, there was a marked increase in the number of microbody profiles and in hepatic catalase activity (16). In the present study, however, in contrast to the situation with CPIB, nafenopin induced more or less similar degrees of microbody proliferation and catalase induction in both sexes of rats and wild type mice. Sex differences in the metabolism of drugs and in the inducibility of microsomal enzyme systems in rodent liver have been established in several instances (35, 36). The reasons for this differential ability of CPIB and nafenopin in inducing microbody proliferation and catalase synthesis in male and female animals can not be stated with certainty from these experiments. Although CPIB and nafenopin are struc-

turally similar hypolipidemic phenolic ethers, there may exist differences in their metabolism. It is possible that the microbody proliferative effect of CPIB is dependent upon excessive anabolic activity, whereas nafenopin may be less dependent, or may possess androgenic activity. The absence of sex difference in response to nafenopin-induced microbody proliferation and catalase synthesis may be due to the fact that this compound is about five times more potent than clofibrate (CPIB) in producing hypotriglyceridemia (23, 24). Further clarification would probably be achieved by evaluation of the dose in relation to reduction of plasma cholesterol and triglyceride levels. It is not known if CPIB, in concentrations greater than 0.25% in diet, would cause microbody proliferation in female rats and mice. This may not occur in view of the finding that the effect on lipids actually decreases when CPIB is given in doses greater than 0.25%. Despite the uncertainty in understanding the basic difference(s) in the action of these drugs in inducing microbody proliferation in one or both sexes of rats and mice, the present study clearly identifies nafenopin as an effective inducer of microbody proliferation and of catalase synthesis in both sexes.

In contrast to a sex difference in response to CPIB in rats and mice cited above, no such difference was observed in acatalasemic "mutant" mice (17). Both male and female acatalasemic mice given CPIB showed profound increase in number of hepatic microbody profiles. This may be in part due to the genetic makeup of this mutant mouse strain, because, within a given species, genetic variation may be important in determining the occurrence or magnitude of enzyme induction (35). Microbody proliferative response in both sexes of acatalasemic mice after nafenopin was identical to that obtained after the administration of CPIB.

The increase in catalase activity and in the amount of catalase protein in liver of rats treated either with nafenopin or with CPIB was twice that of controls. It is of interest to note that the induced steady state of liver catalase in rats is never more than twofold. Furthermore, even in animals given diets containing both nafenopin and CPIB, the concentration of liver catalase did not exceed this level.[2] The amount of liver catalase protein in these animals, therefore, does not

[2] Reddy, J. Unpublished observations.

correspond with the severalfold increase in the number of microbody profiles. Although, cytochemically, catalase was demonstrable in all the microbody profiles, the possibility that these structures proliferating under the influence of nafenopin or CPIB (37) possess a low concentration of catalase cannot be excluded. This is not surprising in view of our earlier observation that CPIB induces hepatic microbody proliferation in animals treated with allylisopropylacetamide, a drug which completely inhibits the catalase synthesis (18).

The relationship between the increase in the number of hepatic microbody profiles and decrease in serum cholesterol and/or triglycerides resulting from the administration of CPIB and nafenopin, is not clear. Despite numerous investigations, the mechanism by which CPIB elicits the hypolipidemic and microbody proliferative responses cannot be stated with certainty. Since nafenopin is a relatively new compound in the expanding field of hypolipidemic agents, studies dealing with the mechanism of its action are lacking, because it was withdrawn from clinical studies after it was found to produce liver tumors in mice.[3] Reduction in serum glycerides and induction of hepatic microbody proliferation and of catalase appear to be the common properties of these two drugs. Because of the significant increase in microbody population and in catalase activity resulting from CPIB, Svoboda and Azarnoff (14) invoked a possible relationship between CPIB-induced microbody proliferation and hypolipidemia. A similar relationship between microbodies and lipid metabolism was suggested earlier by Novikoff and Shin (12) on the basis of the observation of Hochstein and Ernster (38) that the rat liver microsomes catalyze lipid peroxidation that can lead to the production of hydrogen peroxide.

Administration of bovine hepatic catalase has been shown to induce a hypocholesterolemic response in humans and rabbits (39, 40). More recently, Goldfischer et al. (31) showed that in acatalasemic mouse mutants the serum cholesterol and triglycerides are significantly lower, suggesting that in vivo peroxidase is responsible for hypolipidemia. A definite relationship between in vivo peroxidase and hypolipidemia in acatalasemic mice appears doubtful because of the recent

[3] Wagner, W. E. CIBA Pharmaceuticals, personal communication.

demonstration of an abnormality of hepatic lipo-genesis in this mutant strain (41).

In view of the reports dealing with the effect of catalase on cholesterol, it appeared reasonable to assume that the hypolipidemic effect of CPIB was the result of endogenous elevation of liver catalase produced by microbody proliferation (14). How-ever, accumulated evidence indicates that the hypolipidemic and microbody proliferative effects of CPIB are possibly independent (15, 16, 18, and 42). In the present study, the demonstration of microbody proliferation and catalase induction after administration of nafenopin, a potent hypo-lipidemic drug, again raises the question of possible relationship between microbody proliferation and hypolipidemia. In this regard, it is also pertinent to state that methyl clofenapate (43), a hypo-lipidemic analogue of CPIB, also causes a very significant hepatic microbody proliferation. On an equivalent weight basis, methyl clofenapate appears to be 5–10 times as potent as nafenopin or CPIB in inducing microbody proliferation. These drugs promise to be of considerable value for studying the functional significance of micro-body proliferation in the production of hypo-chlesterolemia and hypotriglyceridemia. Further-more, acetylsalicylic acid which induces micro-body proliferation is also known to reduce serum cholesterol levels (42). It is, therefore, necessary to investigate the structural analogues of CPIB in an attempt to determine the interrelationships of chemical configuration of these drugs, microbody proliferation, catalase induction, and hypolipi-demia.

Although the role of drug-induced hepatic microbody proliferation is not understood, the widespread occurrence of microbodies in several cell types of vertebrates has given rise to addi-tional speculation regarding their functional significance and has been the subject of discussion in a recent symposium.[4] In view of their wide-spread distribution among tissues and species, the possibility that microbodies may perform different functions in different cell types cannot be excluded, although their role in lipid metabo-lism and in detoxification of H_2O_2 may be con-sidered universal (13).

The observations on microbody profiles during

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[4] The symposium on "peroxisomes" organized by The Histochemical Society on April 14, 1973, at its 24th annual meeting held in Atlantic City, N. J.

recovery after withdrawal of nafenopin were similar to those seen in rats after CPIB (44). Dis-solution of the microbody matrix, appearance of nucleoid cores in dilated endoplasmic reticulum membranes or occasionally in the hyaloplasm, are the significant findings. Incorporation of microbody profiles into lysosomes or into auto-phagocytic vacuoles was not detected. It appears that cessation of enhanced synthesis of peroxisomal proteins after discontinuation of nafenopin is responsible for the reduction in the peroxisomal protein pool. The reduction in number of micro-body profiles appears to be due to progressively diminishing quantities of peroxisomal matrix proteins in endoplasmic reticulum channels.

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