Hepatic Microbody Proliferation and Catalase Synthesis Induced by Methyl Clofenapate, a Hypolipidemic Analog of CPIB

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The effects of the administration of methyl clofenapate (methyl-2-[4-(*p*-chlorophenyl)phenoxy]2-methylpropionate) on the inducibility of hepatic microbody (peroxisome) proliferation and catalase synthesis were studied in male rats and in both sexes of wild type (Cs^a strain) and acatalasemic (Cs^b strain) mice. These investigations included electron microscopic examination of livers, assay of liver catalase activity, quantitation of catalase protein by immunotitration procedure, and measurements of serum cholesterol and glyceride-glycerol levels. In all groups of animals administration of methyl clofenapate at dietary concentrations of 0.015, 0.05 and 0.125% produced a significant and sustained increase in number of hepatic microbody (peroxisome) profiles. There was no appreciable increase in mitochondrial population, but several mitochondria were markedly enlarged and possessed numerous cristae. The hepatic microbody proliferation in male rats and in both sexes of wild type mice following methyl clofenapate administration was associated with a twofold increase in catalase activity and in the concentration of catalase protein. The increase in microbody population in acatalasemic mice, however, was not accompanied by a significant elevation of the catalase activity, which is due to the unusual heat lability of the mutant catalase enzyme. A marked decrease in serum cholesterol and glyceride-glycerol levels was observed in male rats following methyl clofenapate administration which paralleled the increase in liver catalase activity. In both strains of mice there was a significant reduction in serum glyceride-glycerol concentrations. All the above effects of methyl clofenapate were fully reversed when the drug was withdrawn from the diet of male wild type mice. The demonstration of microbody proliferation and catalase induction with hypolipidemic compounds, CPIB, nafenopin and, in these studies, with methyl clofenapate suggests a possible but as yet unclarified relationship between microbodies and hypolipidemia (Am J Pathol 75:103–118, 1974).

STUDIES WITH ETHYL-α-*p*-CHLOROPHENOXYISOBUTYRATE (CPIB, Atromid®-S), a potent hypocholesterolemic and hypotriglyceridemic drug, have demonstrated convincingly that this compound is an effective inducer of hepatic catalase (hydrogen peroxide-hydrogen peroxide oxidoreductase, EC 1.11.1.6) in male rats and male mice.[1-2] The elevation in liver catalase activity following the administration of CPIB is

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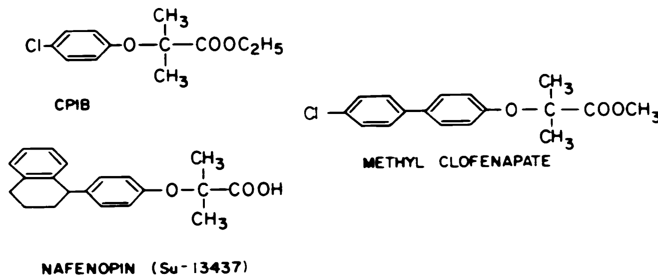
associated with a significant increase in number of microbody (peroxisome) profiles in hepatic parenchymal cells.[1,3-6] It is clearly established by the studies of De Duve and his collaborators that the liver catalase is localized predominantly in microbodies and it accounts for approximately 16% of the total peroxisomal proteins.[7,8] The size, shape and number of microbody (peroxisome) profiles in liver cells following CPIB treatment appear to reflect the amount of peroxisomal (microbody) proteins present in the dilated and tortuous endoplasmic reticulum channels.[9,10]

The significance of CPIB-induced microbody proliferation and hepatic catalase synthesis and their relationship to the hypolipidemia, if any, is not clear. Although the accumulated experimental evidence suggests that the microbody proliferative and hypolipidemic properties of CPIB are independent,[11-13] additional studies are warranted to examine the interrelationships of microbody proliferation, catalase synthesis and hypolipidemia because of the recent demonstration of marked proliferation of microbody profiles in both sexes of rats and mice following the administration of another hypolipidemic compound nafenopin (2-methyl-2[*p*-(1,2,3,4-tetrahydro-1-naphthyl)phenoxy]-propionic acid; Su-13437) which is structurally related to CPIB.[14] The present study examines the possibility of hepatic microbody proliferation in rats and mice following the administration of methyl clofenapate (methyl-2-[4-(p-chlorophenyl)phenoxy]-2-methylpropionate; ICI. 55, 695),[15] in view of the recent report that this compound resembles CPIB in eliciting the hepatic catalase and glycerol phosphate dehydrogenase activities.[16] Methyl clofenapate is a closely related hypolipidemic analog of CPIB and the results of the present investigation indicate that on an equivalent weight basis this compound appears to be several times more effective than CPIB and nafenopin in inducing hypolipidemia, microbody proliferation and catalase synthesis. The chemical structure of this compound is compared with CPIB and nafenopin in Text-figure 1.

Materials and Methods

Animals and Administration of the Drug

Inbred male F-344 rats weighing 130 to 150 g were obtained from Simonson Laboratories Inc, Gilroy, Calif. The wild type (Cs^a strain) mice and the acatalasemic (Cs^b strain) mice [17] were from the stock colony of this laboratory derived from mice originally obtained through the generous cooperation of Dr. R. N. Feinstein, Argonne National Laboratory, Argonne, Ill. Methyl clofenapate (generous gift from Dr. J. M. Thorp, Pharmaceutical Division, Imperial Chemical Industries Ltd, Alderley Park, Macclesfield, Cheshire, U. K.) was added to the



TEXT-FIG 1—Structural formulas of CPIB (ethyl-α-*p*-chlorophenoxyisobutyrate), nafenopin (2-methyl-2[P-(1,2,3,4-tetrahydro-1-naphthyl)phenoxy]-propionic acid) and methyl clofenapate (methyl-2-[4-(P-chlorophenyl)phenoxy]-2-methyl propionate).

ground chow in concentrations of 0.015%, 0.05% and 0.125% (w/w) and administered *ad libitum*. Three to 4 animals were used for each dose level and killed at the end of 3 weeks. Animals kept on the normal diet were killed as controls. The reversal of methyl clofenapate-induced microbody proliferation and catalase activity was investigated in male wild type mice only. After receiving 0.05% methyl clofenapate for 3 weeks, 2 animals were killed on 2, 4 and 7 days after withdrawal of the drug from the diet for morphologic studies and liver catalase assay.

Electron Microscopy

For electron microscopic studies, small segments of liver from normal and drug treated animals were obtained following sacrifice or after laparotomy at selected intervals and fixed for 1 to 2 hours at 0 to 4 C in 2% osmium tetroxide buffered with *s*-collidine to pH 7.4. After fixation the tissues were dehydrated in graded series of alcohols and embedded in epoxy resins. Thin sections were cut on an ultramicrotome, stained with lead hydroxide [18] and examined in an electron microscope.

Assay of Liver Catalase Activity

The activity of catalase was determined spectrophotometrically at 25 C as described by Lück [19] on 5% liver homogenates treated with deoxycholate, prepared according to the method of Ganschow and Schimke.[20] Total proteins were measured by Folin's reagent by the method described by Lowry *et al.*[21]

Quantitation of Catalase Protein

The content of liver catalase protein in control and methyl clofenapate treated male rats and wild type mice was determined by the immunotitration method using antibody specific for catalase protein.[20] The anticatalase sera specific for rat and mouse liver catalase was obtained by injecting purified catalase into foot pads of rabbits as described previously.[2]

The immunotitration procedure consisted of addition of increasing amounts of anticatalase serum to constant quantities of 5% liver extract treated with deoxycholate. The mixtures were then incubated at 37 C for 30 minutes and then kept at 4 C for 24 hours. The resulting immunoprecipitates were sedimented, and the catalase activity remaining in the supernatant was assayed. The amount of anticatalase serum required to precipitate completely the catalase protein was calcu-

lated by extrapolations of the linear portions of the titration curves on the graph to zero catalase activity.[2,14,20]

Determination of Serum Cholesterol and Glyceride-Glycerol

Following light ether anesthesia, blood was collected from the abdominal aorta between 9 and 10:00 AM. Serum total cholesterol and glyceride-glycerol levels were estimated after quantitative separation on silicic acid from phospholipids according to the method described by Azarnoff.[22] The changes in both cholesterol and glyceride-glycerol levels were studied in control and methyl clofenapate-treated rats, but in mice the study was limited to glyceride-glycerol levels because of insufficient plasma for determination of total cholesterol.

Results

Microbody Proliferation

In both sexes of wild type mice (Cs^a strain), methyl clofenapate produced a marked increase in liver weights at all dose levels studied (Table 1). Similar increases in liver weights were also noted in both male and female acatalasemic mice (Cs^b strain) after 3 weeks of treatment with the drug (data not presented).

In control wild type and acatalasemic mice, the microbody profiles in liver cells are few in number and are distributed randomly in the cytoplasm (Figure 1). At the end of 1 week of methyl clofenapate adminis-

Table 1—Effect of Methyl Clofenapate on Liver Weight, Liver Catalase Activity and Serum Glyceride-Glycerol Levels of Wild Type (Cs^a Strain) Mice

Group*	No. of animals	Liver weight (g/100 g body wt)	Liver catalase activity (units/mg protein)	Serum glyceride-glycerol (mg/100 ml)
Males				
Control	4	4.9 ± 0.3	39.6 ± 3.4	13.6 ± 2.3
Methyl clofenapate (0.015%)	4	11.8 ± 1.3	76.3 ± 5.1	4.7 ± 0.5
Methyl clofenapate (0.05%)	4	12.7 ± 1.9	91.3 ± 5.8	3.9 ± 1.1
Methyl clofenapate (0.125%)	4	20.3 ± 2.4	79.3 ± 4.8	3.4 ± 0.6
Females				
Control	4	5.1 ± 0.5	38.5 ± 2.8	11.0 ± 1.9
Methyl clofenapate (0.015%)	4	11.8 ± 1.1	73.5 ± 2.6	3.1 ± 1.6
Methyl clofenapate (0.05%)	4	13.3 ± 2.7	74.4 ± 3.9	3.7 ± 1.0
Methyl clofenapate (0.125%)	4	22.1 ± 3.2	77.4 ± 5.2	2.3 ± 0.5

* Methyl clofenapate was added to the ground chow in desired concentrations and fed for 3 weeks. Values are given as mean ± SE.

tration there was a marked increase in the number of microbody profiles in both sexes of wild type and acatalasemic mice which persisted throughout the duration of the drug treatment. The microbody proliferation was very marked with all three dose levels of methyl clofenapate and was found to be of the same degree in male and female mice (Figures 2–4). Considerable variation in size and shape of microbody profiles was evident (Figures 2–4). Several microbody profiles were less than 0.2 μ in diameter and some were larger than 1 μ in diameter. Continuities between two or more adjacent microbody profiles and between microbody profiles and smooth endoplasmic reticulum channels were also encountered frequently. Nucleoids were seen in several microbody profiles of different sizes and shapes. In addition to proliferation of microbody profiles, there was also a significant increase in the vesicles of smooth endoplasmic reticulum.

There was no obvious increase in number of mitochondria in liver cells of both sexes of mice following methyl clofenapate administration. However, several mitochondria were enlarged in size and contained numerous cristate. Irregularities in size and shape of mitochondria were also seen (Figures 3 and 4).

In male rats at 0.015, 0.05 and 0.125%, methyl clofenapate produced marked increase in liver weights (Table 2). Examination of these livers with the electron microscope revealed a significant increase in the number of microbody profiles (Figure 5). The increase in microbody population was comparable to that observed in animals treated with CPIB and nafenopin.[1,14] No significant changes in mitochondrial number or structure were observed in methyl clofenapate-treated rats.

Table 2—Effect of Methyl Clofenapate Treatment on Liver Weight, Liver Catalase Activity and Serum Cholesterol and Serum Glyceride-Glycerol Levels of F-344 Male Rats

Group*	No. of animals	Liver weight (g/100 g body wt)	Liver catalase activity (units/mg protein)	Serum cholesterol (mg/100 ml)	Serum glyceride-glycerol (mg/100 ml)
Control	4	3.9 ± 0.3	37.0 ± 2.8	87 ± 6	13.5 ± 2.5
Methyl clofenapate (0.015%)	3	8.2 ± 0.4	79.8 ± 3.7	40 ± 4	2.8 ± 0.9
Methyl clofenapate (0.05%)	3	8.6 ± 0.7	91.5 ± 6.3	44 ± 3	3.5 ± 0.7
Methyl clofenapate (0.125%)	3	8.3 ± 0.8	84.5 ± 4.9	48 ± 4	2.2 ± 0.4

* Methyl clofenapate was added to the ground chow in desired concentrations and fed for 3 weeks. Values are given as mean ± SE.

Liver Catalase

The results in Tables 1 and 2 indicate that the activity of catalase in the liver increased approximately twofold following methyl clofenapate treatment in both sexes of wild type (Cs^a strain) mice and in male rats. The results in male rats are in agreement with the findings of Krishnakantha and Kurup.[16] The effect of methyl clofenapate on liver catalase levels and microbody population in female rats was not investigated in detail in these studies because of limited supply of this chemical. However, one female rat given 0.05% methyl clofenapate for 3 weeks showed microbody proliferation comparable to that observed in male rat liver. Like nafenopin (Reddy *et al*[23]) methyl clofenapate increased liver catalase activity in both sexes of wild type mice, whereas CPIB did not increase the catalase activity in female mice.[1,5] The increase in liver catalase activity in animals treated with methyl clofenapate is associated with profound increase in number of hepatic microbody profiles.

The liver catalase activity in untreated acatalasemic mutant mice (Cs^b strain) when measured at 25 C was approximately 15 to 20% of the activity observed in wild type mice (Cs^a strain) from which these acatalasemic mutants were derived.[17] Recently it was demonstrated that the catalase enzyme in acatalasemic mice is unusually heat labile and is inactivated rapidly at room temperature.[24] Although there was a profound increase in number of microbody profiles in male and female acatalasemic mice given methyl clofenapate, there was no appreciable increase in liver catalase activity when compared with the untreated controls. The catalase activity in animals given methyl clofenapate was found to be about 11.8 ± 2.3 units/mg protein, whereas in untreated animals the catalase activity was 8.5 ± 1.3 units/mg protein (complete data not presented in tabular form). The low levels of catalase activity in these mutant mice appear to be due to the fact that this enzyme in the present study was assayed at 25 C on liver extracts treated with deoxycholate and kept at room temperature for 30 minutes.[20] However, it is to be pointed out that the amount of liver catalase protein in control acatalasemic mice was shown to be equal to that found in wild type mice and that the nafenopin-induced microbody proliferation was associated with nearly twofold increase in the quantity of catalase protein.[23]

The amount of catalase protein in the liver of methyl clofenapate-treated male rats and wild type mice was determined by the immunotitration method. In male rats treated with 0.05% methyl clofenapate for 3 weeks, the amount of anticatalase serum needed to precipitate catalase from 1 ml of 5% liver extract was found to be 0.26 ml, whereas 0.12 ml of antiserum was required to precipitate the catalase from the same

amount of liver extract of control rat liver. Similar results were obtained in male wild type mice treated with this drug, suggesting that the amount of catalase protein in liver extracts of methyl clofenapate-treated animals is 2.1 times that of the controls. Accordingly, the increase in liver catalase activity in these animals can be attributed to a twofold increase in the amount of catalase protein.

The liver catalase activity, as well as the number of microbody profiles in male Cs^a mice returned to normal levels within 1 week after the withdrawal of methyl clofenapate from the diet.

Serum Cholesterol and Glyceride-Glycerol

A significant reduction in serum cholesterol and serum glyceride-glycerol levels were observed in male rats following the administration of methyl clofenapate (Table 2). In both male and female wild type mice methyl clofenapate caused a marked decrease in serum glyceride-glycerol levels (Table 1). The effect on serum cholesterol levels in this strain of mice was not investigated in the present studies.

Discussion

The results presented in this paper demonstrate that methyl clofenapate, a hypolipidemic analog of ethyl-α-*p*-chlorophenoxyisobutyrate (CPIB), is highly effective in inducing microbody proliferation in the liver cells of both sexes of wild type and acatalasemic mice. A marked increase in microbody population was also observed in hepatic parenchymal cells of male rats. In addition, these studies also indicate that the increase in microbody population is accompanied by a twofold increase in the hepatic catalase activity and in the quantity of catalase protein in male rats and in male and female wild type mice.

Microbodies (peroxisomes) are cytoplasmic constituents which contain catalase, D-amino acid oxidase [D-amino acid-oxygen oxidoreductase (deaminating), EC 1.4.3.3.], urate oxidase (urate-oxygen oxidoreductase, EC 1.7.3.3.) and other oxidative enzymes.[7,8] It appears that liver catalase is almost exclusively localized in microbody profiles. Morphologic studies on CPIB-treated livers strongly indicate that microbody (peroxisomal) proteins accumulate in the dilated and tortuous endoplasmic reticulum channels forming the electron-opaque microbody profiles which display numerous continuities with one another.[9,10] From these observations it appears that microbodies do not exist as individual organelles but merely represent accumulations of peroxisomal proteins in the endoplasmic reticulum channels. On homogenization, the membranes rupture and enclose varying quantities of microbody protein

material and are isolated as particles of different sizes.[9] Recently Krishnakantha and Kurup [16] studied the subcellular localization of catalase in CPIB-treated rat livers by fractionation procedures and observed a significant increase in catalase activity in the cytosol presumably resulting from the rupture of membranes. These findings, as well as the absence of cytochemically demonstrable catalase in hyaloplasm of normal and CPIB-treated livers,[5,6,25] further substantiate the contention that the peroxisomal proteins constitute a common pool which constantly circulate in the endoplasmic reticulum channels.[9,10,26] The marked increase in number of microbody profiles in liver cells of animals following administration of CPIB,[3-6] nafenopin [14] and, in the present studies, methyl clofenapate is believed to be due in part to the induction of catalase protein synthesis and possibly of other peroxisomal proteins. It can be predicted, therefore, that compounds which cause increase in liver catalase activity can also induce microbody proliferation. This situation may be considered analogous to proliferation of smooth endoplasmic reticulum and simultaneous increase in microsomal enzyme activity resulting from phenobarbital administration.[27]

Although quantitative morphometric analysis of the increase of microbodies was not performed in these studies, the preponderance of microbody profiles of different sizes and shapes suggests a several-fold increase in their number following methyl clofenapate administration. The population density of microbodies in the liver cells of both strains of mice treated with methyl clofenapate and controls, however, was estimated by determining the ratio of microbodies to mitochondria in 20 electron micrographs prepared at a final magnification of 8,000 to 15,000. In normal liver cell the ratio between microbodies and mitochondria was 1:5, whereas in the liver cells of mice treated with methyl clofenapate the microbody to mitochondria ratio was 4:1, indicating a profound increase in the number of microbody profiles. The twofold increase in liver catalase, therefore, does not correlate well with the several-fold increase in microbody number. The same discrepancy was noted in animals treated with CPIB and nafenopin, and it was pointed out that the microbodies proliferating under the influence of these drugs possibly possess low concentration of catalase. It is pertinent to note that microbody proliferation can be induced in the absence of significant catalase synthesis,[13] suggesting that the increase in microbody number may in part be due to the synthesis of yet unidentified proteins that makeup between one-half and two-thirds of the total microbody proteins.[8] Alternately, the discrepancy between the numerical increase in microbody profiles and the estimated quantity of catalase protein

may be explained on the basis that the catalase induced by methyl clofenapate a) possesses low specific activity and/or b) that the enzymatic activity is unstable. If the activity of the induced catalase is unstable, it could account for the low levels of catalase protein, since quantitation in these experiments was based on determination of the activity of catalase remaining in the supernatant following immunoprecipitation.[20] Studies are in progress to determine the quantity of catalase protein by simple radial immunodiffusion in plates, a technic not dependent on the assay of enzymatic activity.

Methyl clofenapate is a closely related analog of CPIB and is metabolized *in vivo* to the corresponding acid.[15] On an equivalent weight basis, methyl clofenapate was five to sixteen times more effective than CPIB in causing both microbody proliferation and hypolipidemia. The induction of microbody proliferation, catalase synthesis and hypolipidemia by methyl clofenapate, nafenopin and CPIB provides a good biologic system to verify the possible role of microbody (peroxisomal) enzymes in producing hypocholesterolemia.[4,28] Previous studies have demonstrated that injection of bovine hepatic catalase produces a hypocholesterol response in humans [29] and rabbits.[30] Although it can be assumed that the hypolipidemic effect following the administration of CPIB, nafenopin and methyl clofenapate is the result of elevation *in vivo* of microbody catalase, additional data suggest that the microbody proliferative and hypolipidemic effects of CPIB are possibly independent.[11,12] The growing list of hypolipidemic compounds that are capable of inducing significant microbody proliferation and catalase synthesis, strongly suggests that either microbody catalase or some other microbody protein (enzyme) may be responsible for the hypolipidemia. The finding that the same compounds exert the dual property of stimulating the proliferation of microbodies (including the biosynthesis of catalase) and of decreasing the serum lipids suggests a possible relationship between these two responses.[1–3,14,31] However, the possibility that microbody proliferation and hypolipidemia are two independent actions of these hypolipidemic drugs can not be excluded. Future studies are required to elucidate the nature of these biologic responses.

Increase in the content of mitochondrial protein in the liver by 50 to 100% was reported in rats following the administration of CPIB and clofenapate.[16,32] Ultrastructural examination of livers of CPIB [1,5] and methyl clofenapate-treated animals revealed no significant increase in mitochondrial population (See Figures 3, 5). However, several mitochondria in clofenapate-treated livers were large and elongated, which

to a certain extent could account for an increase in mitochondrial protein.

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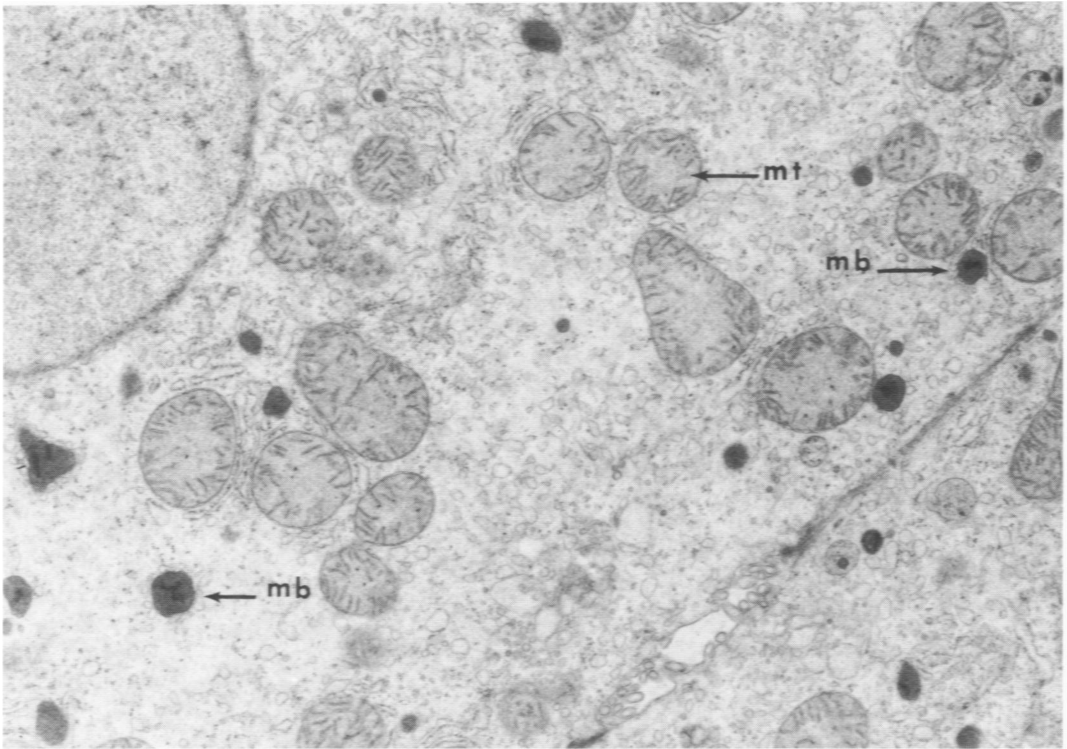
[*Illustrations follow*]



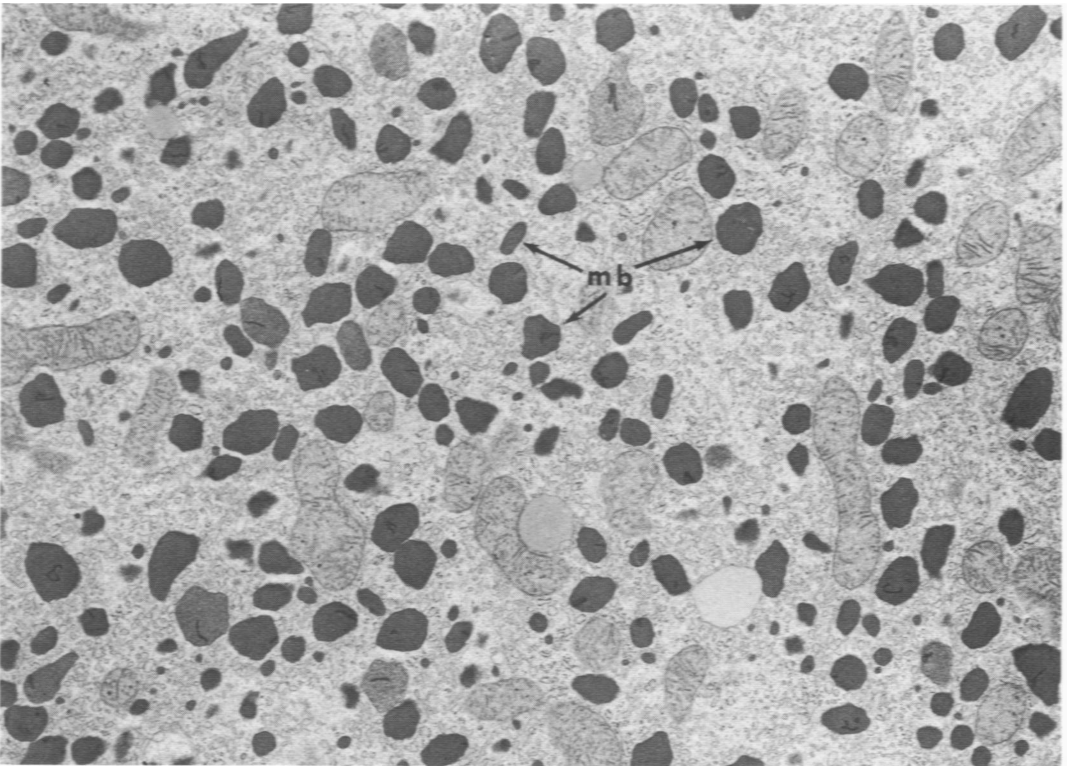
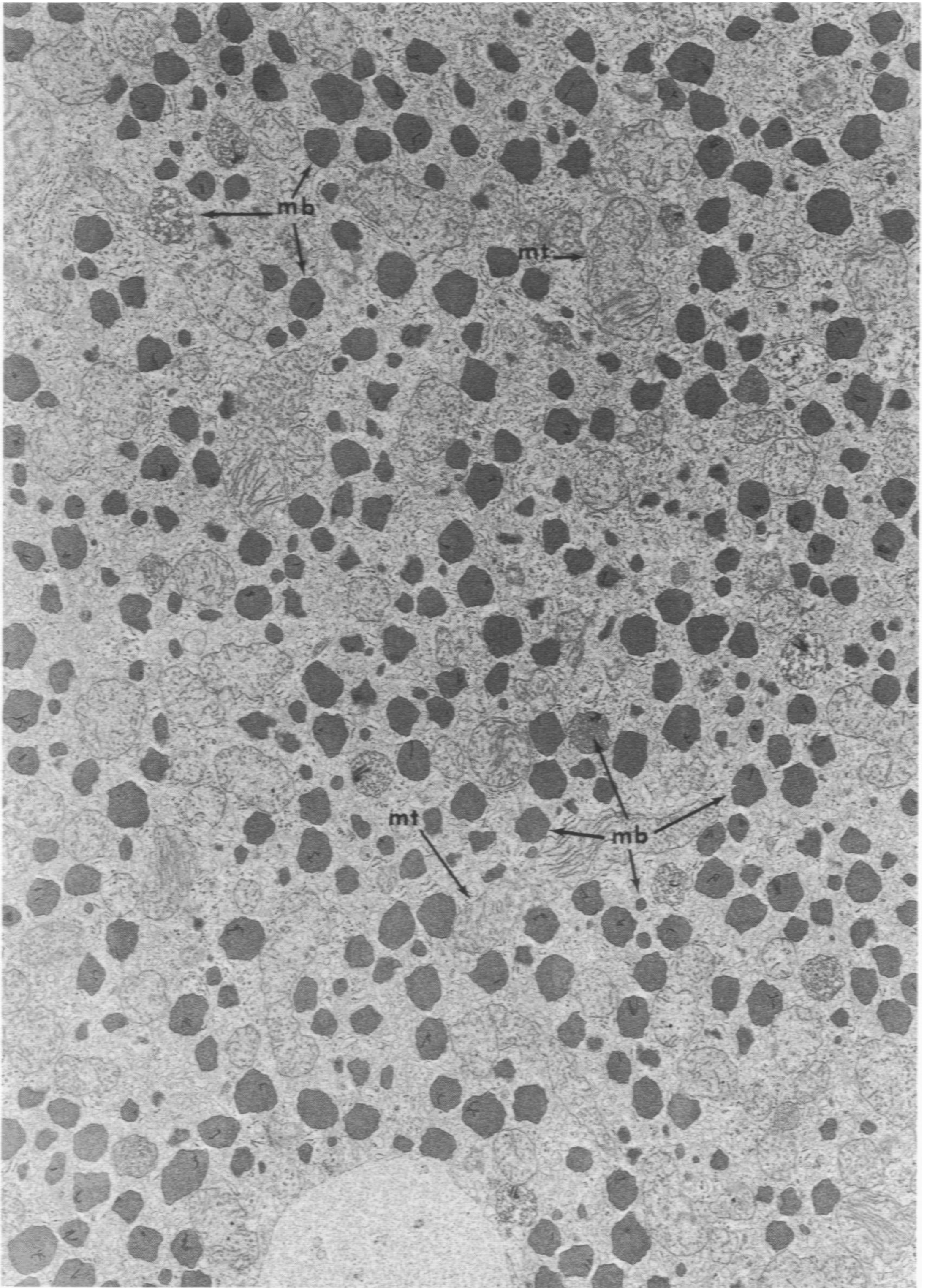


Fig 1—Control male wild type (Cs[a] strain) mouse liver. Few microbody (peroxisome) profiles (*mb*) are seen in the cytoplasm of liver cells. In normal liver cells, mitochondria (*mt*) are numerous in comparison to microbody profiles (× 9500). **Fig 2**—Male wild type mouse given 0.015% methyl clofenapate in the diet for 3 weeks. Numerous microbody profiles (*mb*) are seen in the cytoplasm of liver cells. Marked variation in size and shape of microbody profiles is evident (× 8100).

Fig 3—Female wild type mouse treated with 0.05% methyl clofenapate for 2 weeks. Microbody (*mb*) population is markedly increased and is of the same extent as seen in male mice. Several mitochondria (*mt*) are elongated and show irregularities in shape. There is no obvious increase in number of mitochondria (× 10,200).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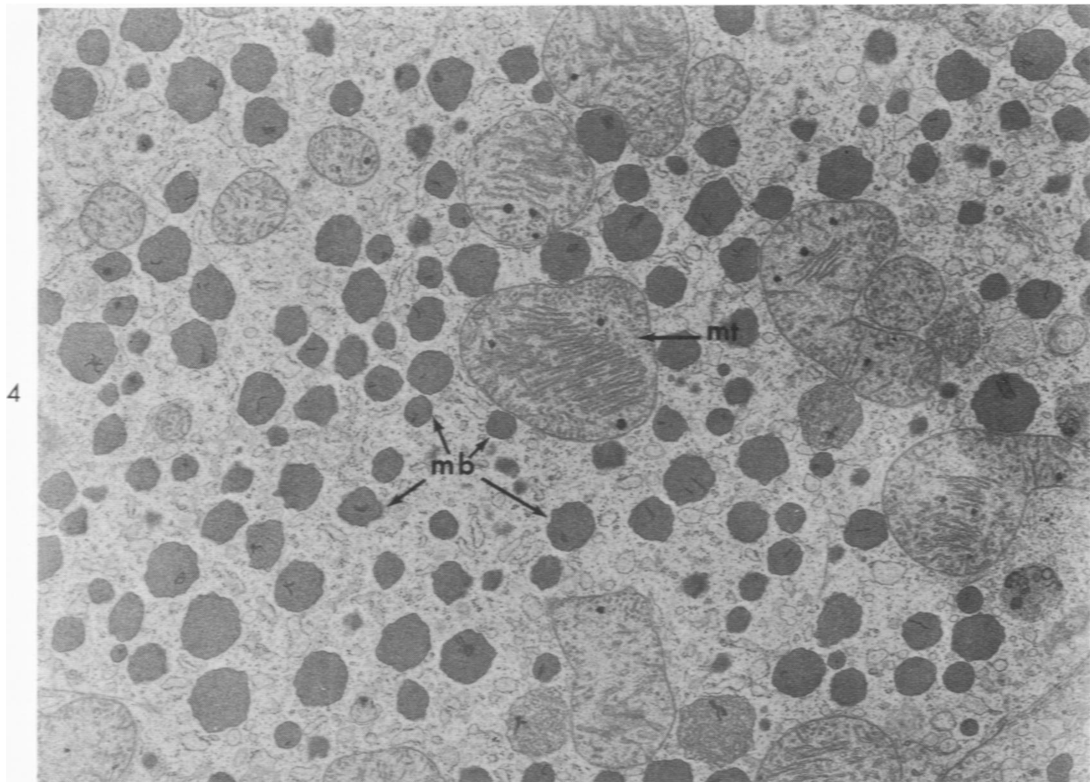

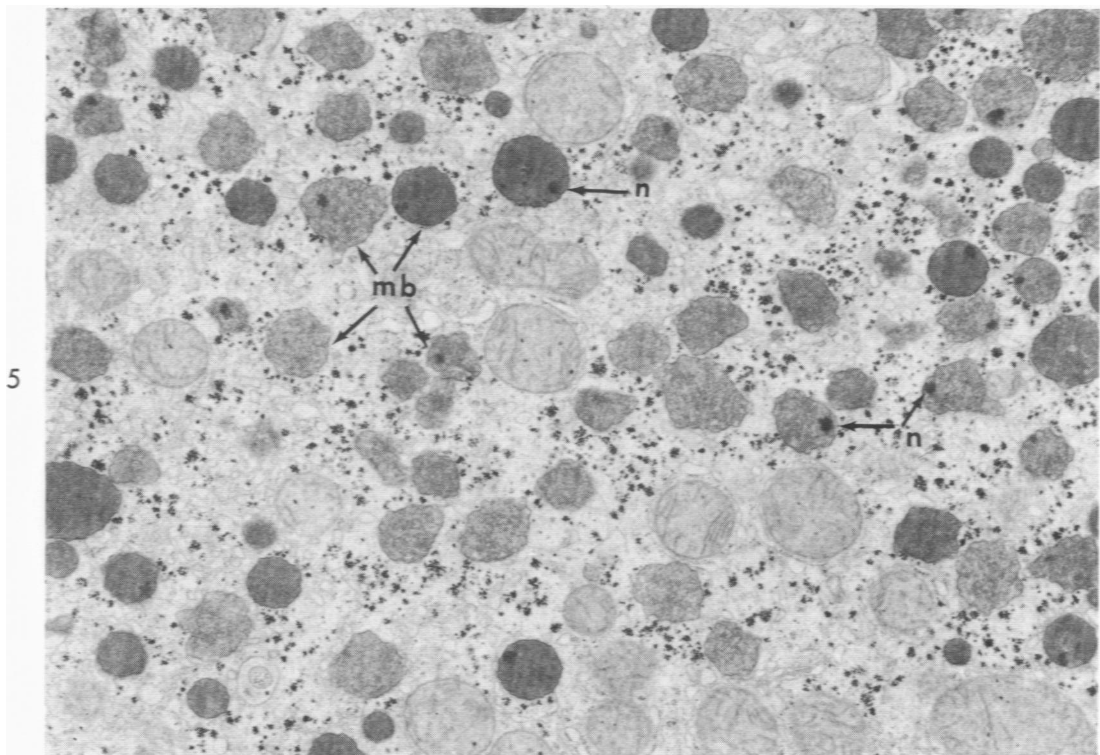

Fig 4—Female acatalasemic (Cs^b strain) mouse. Methyl clofenapate (0.015%) was administered for 3 weeks. Abundant increase in number of microbody (peroxisome) profiles is present. Acatalasemic males given methyl clofenapate also showed marked microbody proliferative response. Mitochondria (*mt*) are large and contain numerous cristae (× 13,000). **Fig 5**—Male rat given 0.125% methyl clofenapate in the diet for 3 weeks. Considerable increase in microbody (*mb*) population was noted in liver cells of male rats given 0.05% and 0.125% dose levels. Uricase-containing nucleoids (*n*) are seen in some microbody profiles (× 14,000).