

## MICROBODIES IN EXPERIMENTALLY ALTERED CELLS

### VII. CPIB-induced Hepatic Microbody Proliferation in the Absence of Significant Catalase Synthesis

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Previous studies (1-3) demonstrated a significant increase in number of microbodies (peroxisomes) and concomitant elevation in catalase activity in male rat liver following administration of ethyl- $\alpha$ -*p*-chlorophenoxyisobutyrate (clofibrate, CPIB), a hypolipidemic drug that lowers serum cholesterol and triglycerides in man (4) and in experimental animals (5, 6). Neither the significance of microbody proliferation induced by CPIB nor the nature of controlling mechanism(s) involved is known, although a possible relation of microbodies to lipid metabolism has been suggested (7). That proliferation of hepatic microbodies in rats is independent of hypolipidemia following CPIB treatment, however, has been shown in studies in which thyroidectomy abolished the

hypolipidemic effect of CPIB but did not interfere with the microbody proliferation and increase in catalase activity (8). Although a close parallelism existed between catalase activity and numerical increase in microbodies in male rat liver following CPIB treatment (3), no such relationship was observed in recent studies on acatalasemic (Cs<sup>b</sup>) mice given CPIB (9). The marked increase in microbodies in the livers of these mutant mice, genetically deficient in catalase (10), was not associated with any significant rise in catalase activity (9). This observation of lack of elevation of catalase, which is one of the principal enzymes of microbodies (11), suggested that microbody proliferation can be induced in rat liver in the absence of significant catalase synthesis.

In the present study, the effect of CPIB on male rat liver was examined in combination with (a) allylisopropylacetamide (AIA) which efficiently blocks the synthesis of catalase (12) without interfering with the activity of previously formed catalase, with (b) aminotriazole which irreversibly inhibits catalase (13) without interfering with its synthesis, and with (c) cycloheximide, a potent inhibitor of protein synthesis (14). The data suggest that microbody proliferation in the rat liver can be achieved by administering CPIB even in the absence of significant catalase synthesis or in the presence of an agent that irreversibly binds with catalase. The numerical increase in microbodies observed under these experimental conditions was attended with negligible catalase activity in the livers. However, the microbody proliferation induced by CPIB was completely abolished by cycloheximide.

#### MATERIALS AND METHODS

Inbred male F-344 rats (A. R. Schmidt Co., Madison, Wisconsin) weighing between 165–200 g were used in these experiments. They were force-fed our standard purified synthetic diet (15) containing 16% casein (vitamin-free), 62.5% sucrose, 10% corn oil, 2.5% vitamin mixture (Nutritional Biochemicals Corporation, Cleveland, Ohio), 4% salt mixture (USP XIV), and 5% alphacel. The animals were fed by stomach tube, 10 g of diet daily, administered in 2 equal doses. This provided approximately 40 cal/day per animal, and was considered to be adequate caloric intake for maintenance of growth (16). The forced-feeding technique was adopted to avoid the possible complication of caloric deficiency, since the animals receiving allylisopropylacetamide (AIA) tended to be slightly drowsy for 1–2 hr following each injection and may not have taken adequate and uniform amounts of a diet if given *ad lib*.

#### *Chemicals, Dose, and Mode of Administration*

The distribution of rats into experimental groups is indicated in Table I. CPIB (Ayerst Laboratories, New York) was administered in a daily dose of 250 mg/kg body weight by stomach tube, along with the diet. Allylisopropylacetamide (AIA) (Hoffman-La Roche, Inc., Nutley, N. J.) dissolved in saline was administered intraperitoneally in a dose of 200 mg/kg body weight every 12 hr. 3-amino-1,2,4-triazole (K & K Laboratories Inc., Plainview, New York) was dissolved in water and injected intraperitoneally (1 gm/kg body weight) twice daily. Cycloheximide (Sigma Chemical Co., St. Louis, Mo.) was given

intraperitoneally in a dose of 0.5 mg/kg body weight every 12 hr.

#### *Morphological and Biochemical Methods*

From all groups, samples of liver were fixed for 1–2 hr in 2% osmium tetroxide buffered to pH 7.4 with S-collidine (2,4,6-trimethylpyridine [Vaughn Inc., Memphis, Tenn.]), and embedded in Epon (15). Sections were cut with glass knives on an LKB ultramicrotome, stained with lead hydroxide (17), and examined with an RCA 3G electron microscope. For catalase determinations, 1 g of liver in 4 ml of M/150 phosphate buffer was homogenized at 1–4°C with 30 strokes in a Potter-Elvehjem homogenizer at 400 rpm. Catalase activity was determined by the spectrophotometric method of Lück (18) after 24 hr of extraction. Total proteins were determined by the method of Lowry et al. (19).

#### RESULTS

Table I shows the alterations in catalase activity and in liver weight in each of the experimental groups. It is evident that the administration of CPIB for 7 days increases markedly the catalase activity in the liver. The liver catalase levels were reduced to less than 5% of normal value, when AIA was administered alone or in combination with CPIB. Administration of AIA for 1 wk prior to giving CPIB (Group V) had a similar effect on the inhibition of catalase activity. The liver weight, expressed as per cent of body weight, increased significantly in animals given CPIB or AIA individually or in combination.

In accord with previous studies (2, 3), done with electron microscopy, significant increase in the number of microbodies was observed in the liver cells of rats given CPIB for 1 wk. Administration of AIA alone produced no obvious signs of liver cell degeneration or necrosis. The most conspicuous alterations were disorganization of the parallel arrays of rough endoplasmic reticulum with focal detachment of ribosomes, depletion of glycogen, and prominent proliferation of smooth endoplasmic reticulum (Figs. 1, 2). Although the mitochondria showed no consistent abnormalities in size or configuration, a few of them had condensations possibly of lipid or protein in their matrix (Fig. 3). The microbodies in the livers of rats treated with AIA alone for 7 days (group III) were somewhat smaller than normal and showed no apparent increase in their number (Fig. 1). The matrix of several microbodies appeared less dense than in controls, and the nucleoids were

TABLE I  
Alterations in Catalase Levels and Liver Weight in F-344 Male Rats

Groups*	No. of animals‡	Liver weight (Mean ± standard error)	Catalase units/mg protein (Mean ± standard error)
		<i>g/100 g body weight</i>	
I Controls, untreated	5	3.8 ± 0.1	40.2 ± 3.8
II CPIB, 7 days	5	6.2 ± 0.2	85.1 ± 5.9
III AIA (200 mg/kg body weight) 2 × daily for 7 days	4	5.4 ± 0.2	3.8 ± 0.5
IV AIA (200 mg/kg body weight) daily + CPIB, 7 days	4	7.1 ± 0.1	4.5 ± 0.4
V AIA (200 mg/kg body weight) 2 × daily for 7 days followed by AIA + CPIB for 7 days	4	8.2 ± 0.3	4.7 ± 0.3
VI Aminotriazole (1 g/kg body weight) 2 × daily, 7 days	3	4.1 ± 0.2	2.9 ± 0.7
VII Aminotriazole (1 g/kg body weight) 2 × daily + CPIB, 7 days	3	5.1 ± 0.1	10.8 ± 2.3
VIII Cycloheximide (0.5 mg/kg body weight) 2 × daily + CPIB, 7 days	3	4.5 ± 0.3	15.1 ± 2.1
IX Cycloheximide (0.5 mg/kg body weight) 2 × daily for 7 days	3	4.4 ± 0.3	14.9 ± 1.1

\* All animals were fed 16% protein diet by stomach tube (see Methods).

‡ Sacrificed 12 hr after the last injection.

somewhat prominent (Fig. 4). An occasional microbody displayed discontinuities in the limiting membrane, probably due to dissolution or rupture.

Despite the absence of significant catalase activity (Table I) in rats given both AIA and CPIB (Groups IV and V), the liver cells showed typical increase in number of microbodies (Fig. 5), comparable to that observed in rats treated with CPIB alone. In addition to the increase in microbody number, there was extensive proliferation of smooth endoplasmic reticulum similar to that observed in the livers of rats treated either with AIA or CPIB alone. Several microbodies were irregular in size and shape but did not differ from those resulting from CPIB treatment alone (2, 3).

The catalase activity in the livers of rats treated with aminotriazole and CPIB for 1 week and sacrificed 12 hr after the last aminotriazole injection (Group VII) was 10.8 units/mg protein. Though aminotriazole binds irreversibly with catalase, it did not inhibit the increase in number of microbodies that typically occurs with CPIB treatment. Administration of cycloheximide prevented almost completely the increase in microbody number

accompanying CPIB treatment. Sections of liver from CPIB-treated rats given cycloheximide were indistinguishable from those from rats given cycloheximide alone; the most conspicuous changes consisted of enlargement of mitochondria and a marked loss of granular endoplasmic reticulum. Catalase activity in these groups was reduced to approximately one-third the normal value.

#### DISCUSSION

Recent studies have demonstrated that in the livers of male rats microbodies were increased in number and altered in size and shape after the administration of CPIB, a hypolipidemic drug (1, 2). That the increase in microbodies, induced by CPIB, was associated with elevated levels of hepatic catalase was shown in subsequent studies (3). Current experiments in our laboratory, utilizing the techniques outlined by Price et al. (20) for determining the kinetics of catalase synthesis and destruction in vivo, indicate that CPIB increases considerably the rate of catalase synthesis without affecting the rate of its degradation

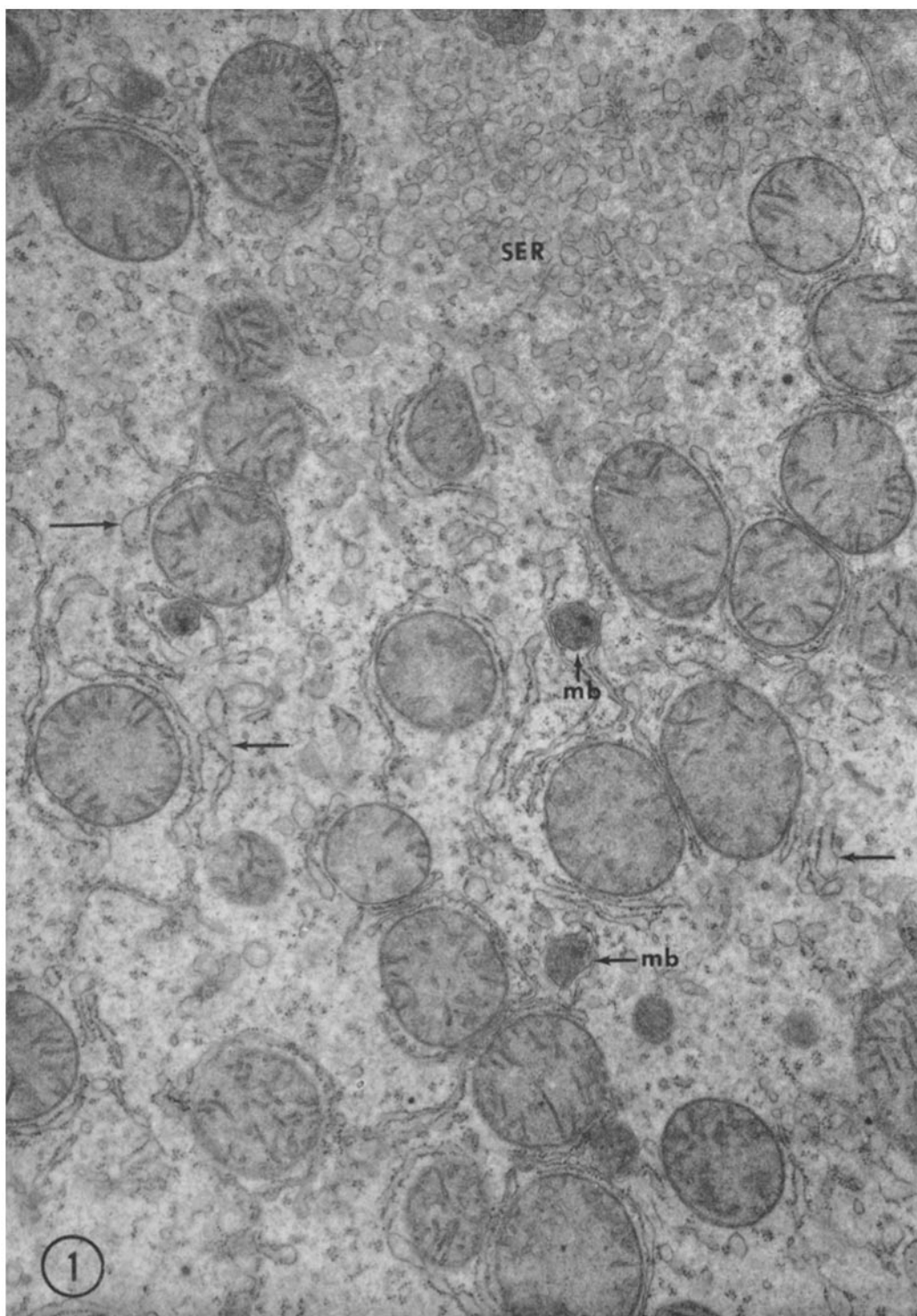
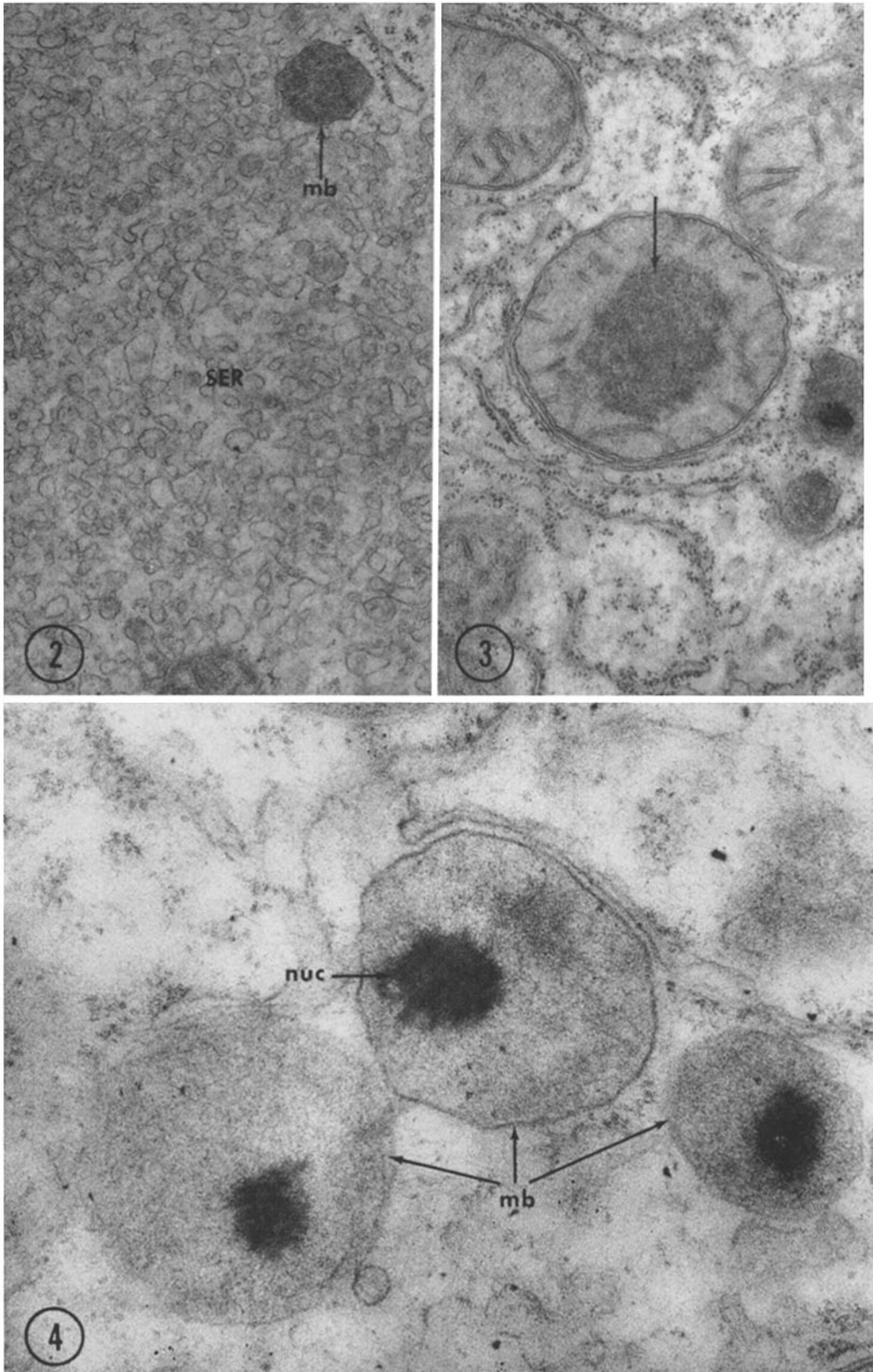


FIGURE 1 Portion of cytoplasm of liver cell of rat treated with allylisopropylacetamide for 7 days. The microbodies (*mb*) are few in number. Single strands of rough endoplasmic reticulum show focal dilatation and detachment of ribosomes (arrows). Proliferation of smooth endoplasmic reticulum (*SER*) was significant.  $\times 20,000$ .



FIGURES 2-4 Male rat liver treated with allylisopropylacetamide for 7 days. Extensive areas of smooth endoplasmic reticulum (*SER*) are present. Some of the mitochondria contain opaque material (Fig. 3, arrow) in the center. The microbodies (*mb*) reveal somewhat lucent matrix and prominent nucleoids (*nuc*) in Fig. 4. Fig. 2,  $\times 40,000$ ; Fig. 3  $\times 50,000$ ; Fig. 4,  $\times 88,000$ .

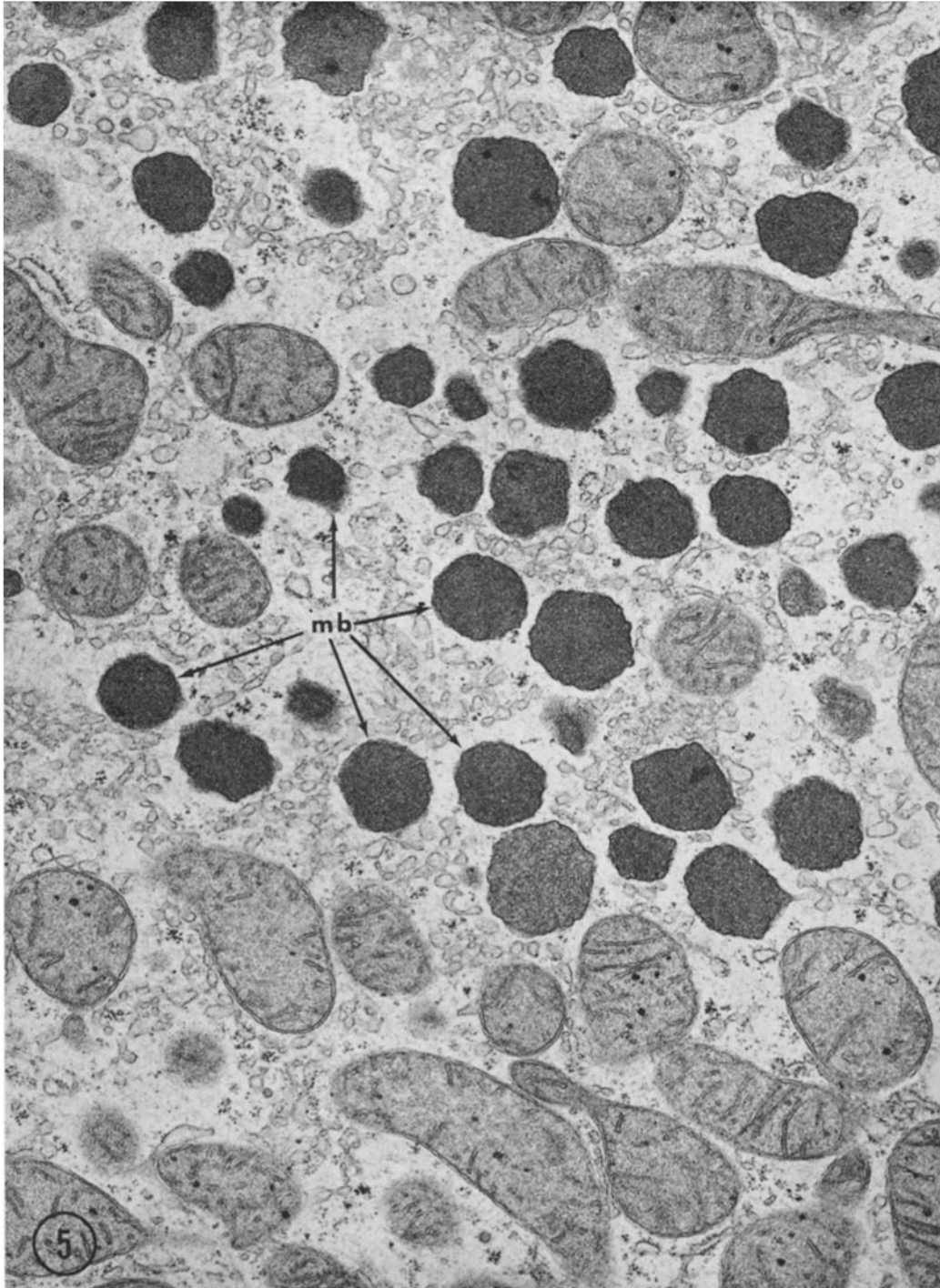


FIGURE 5 Numerous microbodies (*mb*) are observed in the liver cells of rats treated simultaneously with allylisopropylacetamide and CPIB for 7 days.  $\times 16,000$ .

(J. Reddy, M. Chiga, and D. Svoboda, in preparation).

The precise biological significance of microbody proliferation and of increased synthesis of catalase in the liver induced by CPIB and the regulatory mechanism(s) involved in the initiation of these events are, however, not fully elucidated. On the basis of the observation of Hochstein and Ernster (21), Novikoff and Shin (7) suggested a possible relationship of microbodies to lipid metabolism. Since hemoproteins constitute a group of powerful lipid peroxidation catalysts (22), it is possible that the catalase of microbodies has a similar effect and that the microbodies participate in the formation of hydroperoxides of cholesterol esters (23). Under these circumstances, it appeared attractive to formulate a working hypothesis: that the increase in microbody number and catalase activity in the liver is related to the lowering of serum cholesterol levels following CPIB treatment (2, 24). However, this does not appear to be the case since administration of CPIB to thyroidectomized rats resulted in an increase in microbody number and catalase activity (8), although thyroidectomy has been shown to abolish the hypolipidemic action of CPIB (25). From these and other studies (8, 25, 26) it is evident that hepatic microbody proliferation resulting from CPIB treatment is not only independent of the hypolipidemic effect, but may be independent of thyroxine displacement from plasma protein binding sites. Therefore, it is reasonable to assume that hypolipidemia and microbody proliferation are two independent effects of CPIB and that these two effects may not be interrelated.

Because of the temporal association of increase in number of microbodies with elevated levels of hepatic catalase, the increase in microbody formation following CPIB was considered as an expression of enhanced synthesis of catalase enzyme (2), since catalase was reported to constitute 40% of microbody protein (11). Although there is a significant increase in catalase synthesis following the administration of CPIB (Reddy, Chiga, and Svoboda, in preparation), the results of the present investigation, as well as the observations in acatalasemic ( $C_s^b$ ) mice treated with CPIB (9), do not support the contention that the increased microbody population is an expression primarily of the increase in catalase level. In the present studies, when CPIB was administered to rats receiving allylisopropylacetamide (AIA), an agent that

blocks the synthesis of new catalase (20), the increase in microbody number occurred even though catalase activity in the liver was negligible. It is apparent, therefore, that AIA completely inhibits the elevated synthesis of catalase resulting from CPIB treatment, but does not appear to interfere with the increase in microbody formation. Analogous to these findings are the observations in acatalasemic mice treated with CPIB (9). In these mutant mice, genetically deficient in catalase (10), microbody proliferation occurred following the administration of CPIB, but the increase in microbody number was not associated with any increase in catalase activity (9).

De Duve and his associates (11) showed that microbodies possess several enzymes necessary for the production and destruction of hydrogen peroxide and termed then "peroxisomes," to indicate their biochemical potential. In addition to catalase, microbodies were shown to contain several oxidases such as D-amino acid oxidase and L- $\alpha$ -hydroxy acid oxidase. According to Azarnoff and Svoboda (27), while catalase activity is increased significantly in male rats treated with CPIB, there is 80% reduction in activity of D-amino and L- $\alpha$ -hydroxy acid oxidases. Consequently, in the present experiments, the increased number of microbodies in rats treated with AIA and CPIB may also contain minimal amounts of D-amino and L- $\alpha$ -hydroxy acid oxidases. This situation might be identical with that of microbodies of acatalasemic mice treated with CPIB (9). It appears paradoxical, therefore, to have numerous microbodies in the livers of rats treated with AIA and CPIB (also in acatalasemic mice given CPIB) while some of their principal enzymes, catalase and oxidases, are markedly reduced.

Because microbody proliferation can be induced in the absence of significant catalase synthesis and with low levels of oxidases (27; Reddy, Chiga, and Svoboda, in preparation), the view that microbody formation induced by CPIB is due primarily to increased synthesis of catalase enzyme may not be tenable, despite the fact that catalase synthesis as well as total hepatic catalase activity increases markedly with CPIB treatment. The question of what then is the additional triggering mechanism in initiating the microbody proliferation after CPIB treatment remains unanswered.

One possibility is that, in addition to increase in catalase synthesis, CPIB might induce the syn-

thesis of yet unidentified proteins that make up between one-half and two-thirds of the total microbody (peroxisome) proteins, and/or of isocitrate dehydrogenase, which has been shown recently to contribute as much as 25% of the total peroxisome proteins (28). From the present studies, it is evident that cycloheximide, which inhibits protein synthesis by stabilization of polyribosomes (14), abolished completely the microbody proliferation expected from CPIB. The observations of Platt and Cockrill (29) and preliminary studies in this laboratory indicate that the concentration of isocitrate dehydrogenase increases in the livers of rats treated with CPIB; whether this bears any temporal relation to increase in microbody number remains to be investigated. De Duve and Baudhuin (11) suggested that microbodies could participate significantly in the oxidation of cytoplasmic NADH, which is generally oxidized by way of electron shuttles involving an appropriate mitochondrial oxidizing system and the corresponding cell sap dehydrogenase. The increase in

microbody number and of catalase and isocitrate dehydrogenase activity resulting from CPIB treatment suggests possible alterations in the electron shuttles involved in energy production and oxidative metabolism (11).

Another possibility is that microbodies play an essential role in the metabolic degradation of CPIB. This possibility can be examined by using labeled CPIB and studying its incorporation into various cellular organelles by biochemical means and/or by high resolution radioautography.

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