Microbodies in Experimentally Altered Cells

V. Histochemical and Cytochemical Studies on the Livers of Rats and Acatalasemic (Cs^b) Mice Treated with CPIB

Janardan Reddy, MD, Sukhum Bunyaratvej, MD,
and Donald Svoboda, MD

PREVIOUS STUDIES [1-4] have demonstrated that administration of Clofibrate (ethyl-α-p-chlorophenoxyisobutyrate; CPIB) a hypolipidemic drug,[5,6] causes a marked increase in the number of microbodies in the livers of male rats and in both sexes of acatalasemic (Cs^b) mice. Although the majority of the hepatic microbodies induced in rats and mice after CPIB treatment possessed the characteristic central core, or nucleoid, several anucleoid structures resembling microbodies also were observed.[1,2,4] These anucleoid structures in CPIB-treated rat livers were interpreted by Paget [7] as "lysosomes," but were referred to as microbodies in our previous studies [1,2] because of their characteristic size and shape, and the appearance of their matrices. This interpretation appeared consistent with the biochemical findings of increased catalase activity in the livers of CPIB-treated male rats,[2] since catalase constitutes 40% of microbody protein.[8] However, the increase in microbody population, occurring in male and female acatalasemic mice treated with CPIB, was not associated with any significant rise in catalase activity.[4]

In this paper, we report the results of histochemical and cytochemical (peroxidase, acid phosphatase, and urate oxidase) studies on the livers of rats and acatalasemic mice treated with CPIB. The results demonstrate a significant increase in peroxidase-positive organelles in the livers of CPIB-treated animals, and support the previous morphologic studies of increased microbody population.[1,2,4] Furthermore, the numerous anucleoid dense bodies resulting from CPIB treatment were peroxidase-positive and acid phosphatase-negative, thus excluding the possibility of their being lysosomes.

From the Department of Pathology and Oncology, University of Kansas Medical Center, Kansas City.

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Address for reprint requests: Dr. Janardan Reddy, Department of Pathology and Oncology, University of Kansas Medical Center, Rainbow Blvd at 39th St, Kansas City, Kan 66103.

Materials and Methods

Rats (F-344) used in these studies were obtained from A. R. Schmidt Company, Madison, Wis. The acatalasemic mice (Cs^b strain) were from a colony derived from mice originally obtained through the generous cooperation of Dr. Feinstein, Argonne National Laboratories, Argonne, Ill. CPIB was administered in ground Purina Chow in a concentration of 0.25%.[1,2]

For electron microscopy, tissues were fixed for 1–2 hr in 2% osmium tetroxide, buffered with s-collidine, dehydrated in a graded series of alcohols, and embedded in epoxy resins. Thin sections were cut on an LKB ultramicrotome, stained with lead hydroxide,[9] and examined in an RCA 3G electron microscope.

Cytochemical Studies

Peroxidase Method. The livers of normal and CPIB-treated rats and acatalasemic mice were perfused via the portal vein with 1.5% distilled glutaraldehyde in 0.15 M phosphate buffer, pH 7.4, and small pieces of liver were fixed overnight in the same solution. Frozen sections, 40 μ thick, were cut and incubated at 37° C for 30–60 min at pH 7.6 or pH 8.0 in the diaminobenzidine reaction medium of Graham and Karnovsky[10] containing hydrogen peroxide.[11] Controls consisted of incubations in which 0.02 M 3-amino-1,2,4-triazole was added to the medium, or in which either H_2O_2 or 3,3-diaminobenzidine was omitted. Following incubation, the tissues were postfixed in osmium, and processed for electron microscopy as described above.

Acid Phosphatase Method. Acid phosphatase studies were performed on livers fixed for 4 hr at 0–4° C in 4.5% glutaraldehyde in 0.1 M phosphate buffer pH 7.4,[12] and in incubation medium according to Gomori[13] with β-glycerophosphate and lead nitrate at pH 5.0. Controls consisted of incubation of sections in medium lacking glycerophosphate.

Histochemical Demonstration of Uricase Activity. By employing the technique described by Graham and Karnovsky,[14] the hepatic uricase (urate oxidase) activity was studied. Sections 6–8 μ thick were cut from freshly frozen livers, and treated for 10–15 min in ice-cooled acetone prior to incubation, at 37° C under air for 30 min with or without urate as substrate.

Results

Electron Microscopy

Detailed descriptions of the variations in number and ultrastructure of microbodies in rats and in acatalasemic (Cs^b) mice following CPIB treatment appear in previous reports from this laboratory.[1–4,15] An increase in the number of microbodies was first evident in the hepatocytes of male rats and in both sexes of acatalasemic mice after 3 days of CPIB adminisration; it reached a maximum after 4–5 weeks, and was sustained thereafter by continuing CPIB in the diet (Fig 1).

Peroxidase: General Comments. Sections of glutaraldehyde-fixed tissues incubated at pH 8.0 for 30 min showed greater intensity of the reaction product than those incubated at pH 7.6. A longer period of incubation, 45–60 min, was necessary to obtain adequate reaction in the livers incubated at pH 7.6. Fixation of livers of acatalasemic mice

was generally less satisfactory than that of rat livers. Control sections incubated in the absence of diaminobenzidine showed no reaction product. Similarly, when H_2O_2 was omitted from, or 3-amino-1,2,4-triazole was added to, the incubation medium, a reaction usually was not encountered, except in a few peribiliary dense bodies.

Localization in Rats. Reaction product was present in normal rat liver in round and oval structures measuring 0.2–0.8 μ in diameter, located randomly in the cytoplasm. Many of these organelles showing peroxidase reaction were in proximity with rough endoplasmic reticulum, and showed crystalline nucleoids upon higher magnification (Fig 2 and 3). In sections incubated for over 45 min, the majority of the nucleoids, however, could not be visualized because of the masking effect of large amounts of reaction product.

In male rats treated with CPIB, the peroxidase-positive bodies were numerous (Fig 4–7), and corresponded closely to the distribution of microbodies. Several dense bodies, which lacked nucleoids and still were identified as microbodies in previous studies dealing with CPIB,[17,18] consistently gave a positive peroxidase reaction. When sections were incubated for 60 min at pH 8.0, moderate peroxidase reaction was observed in the inner membrane and in a few stacks of cristae of an occasional mitochondrion of the liver. (Fig 6 and 7).

Localization in Acatalasemic Mice. In male and female acatalasemic (Cs^b) mice treated with CPIB, a marked increase in peroxidase-positive organelles was observed (Fig 8 and 9) when compared to controls (Fig 10). These structures varied in size and shape and, to some extent, in the intensity of peroxidase reaction. Because of its delicate filamentous nature, the nucleoid was difficult to visualize in the microbodies after peroxidase staining. Although some of the lysosomes displayed a positive peroxidase reaction, the intensity was clearly of a lesser degree (Fig 9) than in the microbodies. This difference in the intensity of peroxidase reaction was most conspicuous in sections not stained with uranyl acetate or lead, because in unstained sections, peroxidase-positive microbodies stand out prominently.

Acid Phosphatase. In 6- to 8-μ frozen sections of livers of normal as well as CPIB-treated rats and acatalasemic mice, the acid phosphatase activity was localized in the "pericanalicular" regions of hepatic parenchymal cells (Fig 11). Although there was no appreciable increase in acid phosphatase activity during the early stages of CPIB administration, a moderate increase in the activity of the enzyme was noted in animals fed CPIB for more than 5 months. By electron microscopy, the acid phosphatase activity was observed in the "pericanalic-

ular dense bodies," (lysosomes), and occasionally in endoplasmic reticulum and Golgi cisternae. No reaction product was seen in the several anucleoid dense bodies, presumably CPIB-induced microbodies (Fig 12 and 13), appearing in the livers of male rats and in both sexes of acatalasemic mice.

Histochemical Localization of Uricase

Application of the method described by Graham and Karnovsky [14] for the histochemical demonstration of uricase resulted in discrete, reddish-brown, granular deposits of reaction product scattered throughout the cytoplasm of the parenchymal cells of liver in rats and acatalasemic mice. The distribution of uricase activity in liver of male rats and acatalasemic mice treated with CPIB is shown in Fig 14 and 15, respectively, and is clearly different from that of acid phosphatase activity (Fig 11). Numerous granules with reaction product were evident in the hepatocytes of CPIB-treated male rats and in both sexes of acatalasemic mice, and were more abundant than in their corresponding controls.

Discussion

In male rats [1-3] and in both sexes of acatalasemic (Cs^b) mice,[4] an appreciable increase in the number of microbodies was observed following treatment with CPIB, a hypolipidemic drug which lowers serum cholesterol and triglycerides in man [6] and in experimental animals.[5] Since several of the CPIB-induced organelles in these livers lacked the typical central core, or nucleoid, it was essential to characterize their true nature by cytochemical methods.

A cytochemical method for ultrastructural localization of injected horseradish peroxidase was described by Graham and Karnovsky.[10] By using a modification of this method, Novikoff and Goldfisher [16] successfully demonstrated by light and electron microscopy the endogenous peroxidase activity in the hepatic and renal microbodies. Fahimi [11,17] and Essner [18] later used the method for demonstration of microbodies in adult rat and fetal mouse livers. Although the precise mechanism of diaminobenzidine oxidation is not clear, it is attributed to the peroxidatic reaction of microbody catalase,[17] since this enzyme possesses both peroxidatic and catalatic function.[8]

The results of the present studies on the peroxidase activity in liver cells of normal rats are in agreement with the findings of Novikoff and Goldfischer [16] and of Fahimi.[11,17] Reaction product generally was limited to organelles identifiable as microbodies by the presence of

nucleoids. In the livers of control acatalasemic mice, few peroxidase-positive structures were seen. In this investigation, peroxidase studies on CPIB-treated male rats and on both sexes of acatalasemic mice showed a significant increase in the number of peroxidase-positive organelles. These findings clearly substantiate the previous observation of an increase in microbody population, since the typical reaction product was visualized in nucleoid-bearing, as well as anucleoid, microbodies. Furthermore, these organelles did not reveal any acid phosphatase activity when incubated in the Gomori medium,[18] thus excluding the possibility of anucleoid structures being lysosomal in nature.[19]

It was evident in earlier studies[20] that in CPIB-treated male rats, while catalase activity was increased significantly, there was a decrease in activity of D-amino and L-α-hydroxy acid oxidases, suggesting that a heterogenous population of microbodies may arise following CPIB treatment. The possibility that all these organelles might at least contain catalase can be supported by the fact that peroxidatic activity was observed uniformly in all of them, and also by the biochemical studies that showed an increase in catalase activity in male rats treated with CPIB. In contrast, however, no increase in catalase activity was detected in male or female acatalasemic mice given CPIB,[4] although a substantial increase in number of peroxidase-positive microbodies occurred. If it is assumed that the microbody catalase alone is responsible for the peroxidase reaction,[17] the pre-existing low levels of catalase in livers of acatalasemic mice[21] might be adequate to account for the marked peroxidase reaction obtained in innumerable microbodies resulting from CPIB treatment. In such case, one has to suppose further that the catalase present in pre-existing microbodies may be redistributed in the newly formed microbodies resulting from CPIB treatment, and thus be responsible for the peroxidase reaction. Novikoff and Shinn[22] suggested that electron-opaque material may be deposited in the cavities of dilated smooth endoplasmic reticulum before the cavities separate from the main reticulum. Recently, several investigators[23,24] studied the development of microbodies in fetal rat and mouse liver and suggested that microbodies originated from granular endoplasmic reticulum. If it is conceived that the microbodies are in fact bulges that form at the terminal end of certain areas of endoplasmic reticulum,[22] the connection of microbodies with membranes of endoplasmic reticulum[1,2] and the occasional continuity between two microbodies in CPIB-treated animals could constitute the channel for redistribution of catalase. Since the mechanism of diaminobenzidine oxidation is not

understood, it is plausible to assume that other factors, in addition to catalase, may be involved.

Uricase Activity. The results of histochemical studies of uricase activity in normal rats are in agreement with the findings of Graham and Karnovsky.[18] In the present studies, significant increase in uricase activity was observed histochemically in livers of male rats and in male and females acatalasemic mice after CPIB treatment. These findings are in accord with the intrastructural studies showing an increase in the number of microbodies. Since there is no available method for demonstrating uricase activity at the ultrastructural level, it is not known whether uricase activity is present in the anucleoid microbodies.

Summary

A significant increase in the number of hepatic microbodies, several of which lacked nucleoids, was demonstrated in previous studies in male rats and in acatalasemic (Cs^b) mice treated with CPIB, a hypolipidemic drug. In the present studies, the peroxidase, acid phosphatase, and urate oxidase activities were investigated by histochemical and cytochemical methods. A marked increase in the number of peroxidase-positive organelles, corresponding to the distribution of microbodies, was observed in the livers of male rats and of both sexes of acatalasemic mice treated with CPIB. Numerous anucleoid dense bodies resulting from CPIB treatment could be identified positively as microbodies since they were peroxidase-positive and acid phosphatase-negative. A moderate increase in urate oxidase activity in the liver also was seen in the histochemical studies in the animals treated with CPIB.

References

1. SVOBODA, D. J., and AZARNOFF, D. L. Response of hepatic microbodies to a hypolipidemic agent, ethyl chlorophenoxyisobutyrate (CPIB). *J Cell Biol* 30:442–450, 1966.
2. SVOBODA, D., GRADY, H., and AZARNOFF, D. Microbodies in experimentally altered cells. *J Cell Biol* 35:127–152, 1967.
3. SVOBODA, D., AZARNOFF, D., and REDDY, J. Microbodies in experimentally altered cells. II. The relationship of microbody proliferation to endocrine glands. *J Cell Biol* 40:734–746, 1969.
4. REDDY, J., BUNYARATVEJ, S., and SVOBODA, D. Microbodies in experimentally altered cells. IV. Acatalasemic (Cs^b) mice treated with CPIB. *J Cell Biol* 42:587–596, 1969.
5. AZARNOFF, D. L., TUCKER, D. R., and BARR, G. A. Studies with ethyl chlorophenoxyisobutyrate (clofibrate). *Metabolism* 14:959–965, 1965.
6. OLIVER, M. F. Further observations on the effects of Atromid and of ethyl chlorophenoxyisobutyrate on serum lipid levels. *J Atheroscler Res* 3:427–444, 1963.

7. PAGET, G. E. Experimental studies of the toxicity of Atromid with particular reference to fine structural changes in the livers of rodents. *J Atheroscler Res* 3:729–736, 1963.

8. DE DUVE, C., and BAUDHUIN, P. Peroxisomes (microbodies and related particles). *Physiol Rev* 46:323–357, 1966.

9. KARNOVSKY, M. J. Simple methods for "staining with lead" at high pH in electron microscopy. *J Biophys Biochem Cytol* 11:729–732, 1961.

10. GRAHAM, R. C., JR., and KARNOVSKY, M. J. The early stages of absorption of injected horseradish peroxidase in the proximal tubules of mouse kidney: Ultrastructural cytochemistry by a new technique. *J Histochem Cytochem* 14:291–302, 1966.

11. FAHIMI, H. D. Cytochemical localization of peroxidase activity in rat hepatic microbodies (peroxisomes). *J Histochem Cytochem* 16:547–550, 1968.

12. SABATINI, D. D., BENSCH, K., and BARRNETT, R. J. Cytochemistry and electron microscopy. The preservation of cellular ultrastructure and enzymatic activity by aldehyde fixation. *J Cell Biol* 17:19–58, 1963.

13. GOMORI, G. W. *Microscopic Histochemistry: Principles and Practice*. Univ. Chicago Press, Chicago, 1952.

14. GRAHAM, R. C., JR., and KARNOVSKY, M. J. The histochemical demonstration of uricase activity. *J Histochem Cytochem* 13:448–453, 1965.

15. SVOBODA, D., REDDY, J., and AZARNOFF, D. Microbodies in experimentally altered cells. III. Responses of matrix and nucleoids. *J Ultrastruct Res* In press.

16. NOVIKOFF, A. B., and GOLDFISCHER, S. Visualization of microbodies for light and electron microscopy. *J Histochem Cytochem* 16:507, 1968.

17. FAHIMI, H. D. Peroxidase activity of microbody catalase. *J Cell Biol* 39:42A, 1968.

18. ESSNER, E. Cytochemical studies of microbodies in developing hepatocytes. *J Cell Biol* 39:42A, 1968.

19. NOVIKOFF, A. B. "Lysosomes in the Physiology and Pathology of Cells: Contributions of Staining Methods." In *Ciba Foundation Symposium on Lysosomes*. DE REUCK, A. V. S., and CAMERON, M. P., Eds. Little, Boston, 1963, p. 36.

20. AZARNOFF, D., and SVOBODA, D. Changes in microbodies in the rat induced by ethyl-p-chlorophenoxyisobutyrate. *J Lab Clin Med* 68:854, 1966.

21. FEINSTEIN, R. N., BRAUN, J. T., and HOWARD, J. B. Acatalasemic and hypocathalasemic mouse mutants. II. Mutational variations in blood and solid tissue catalases. *Arch Biochem* 120:165–169, 1967.

22. NOVIKOFF, A. B., and SHINN, W. Y. The endoplasmic reticulum in the Golgi zone and its relations to microbodies, Golgi apparatus and autophagic vacuoles in rat liver cells. *J Micr* 3:187–206, 1964.

23. ESSNER, E. Endoplasmic reticulum and the origin of microbodies in fetal mouse liver. *Lab Invest* 17:71–87, 1967.

24. TSUKADA, H., MOCHIZUKI, Y., and KONISHI, T. Morphogenesis and development of microbodies of hepatocytes of rats during pre- and postnatal growth. *J Cell Biol* 37:231–243, 1968.

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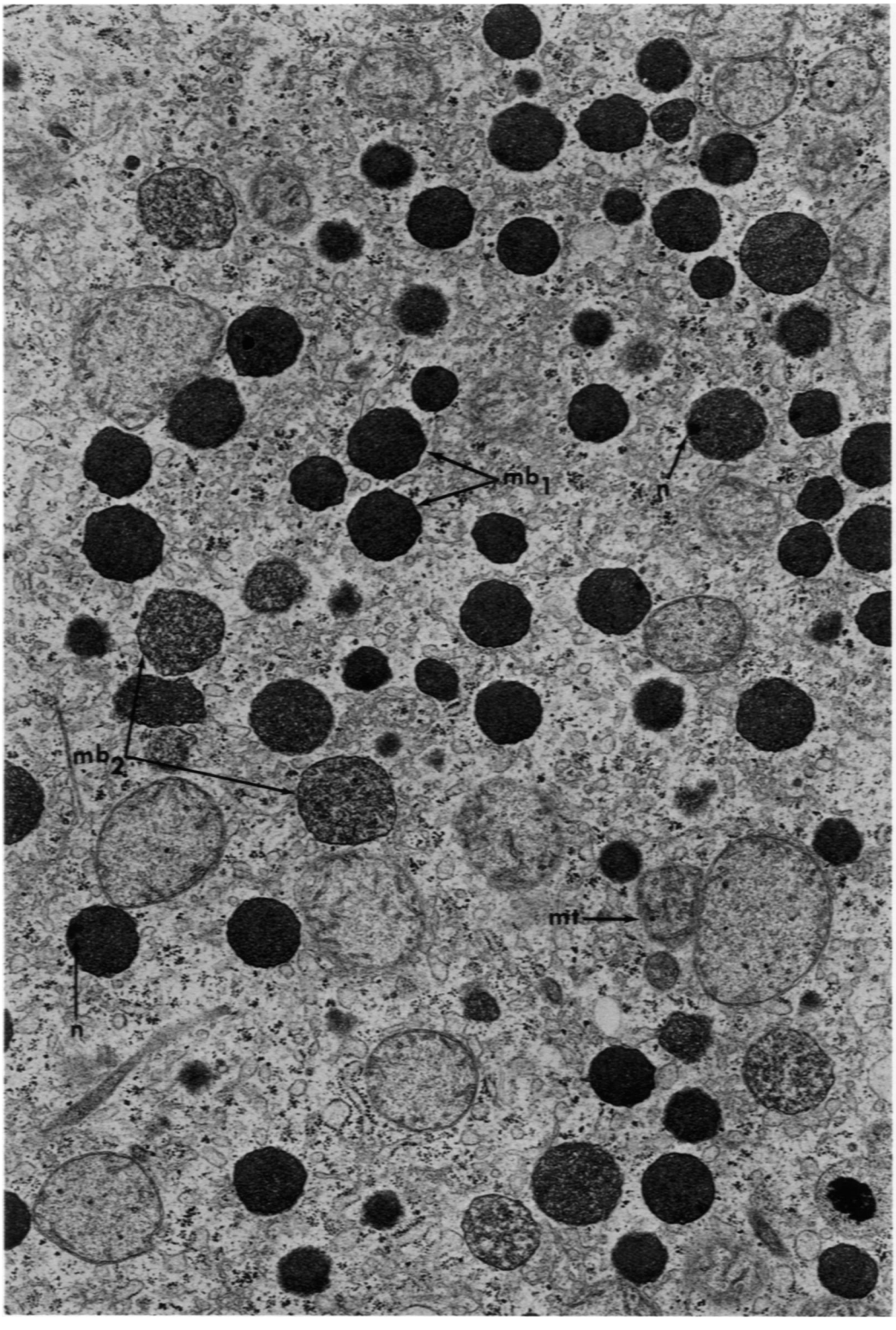
Legends for Figures

Fig 1. Male rat liver, 3 weeks after CPIB administration. Liver cells contain numerous microbodies with typically dense matrix (*mb_1*), while others display pale matrix (*mb_2*). Several microbodies in this figure do not contain a nucleoid (*n*), although it can be visualized in some. Mitochondria (*mt*). × 11,000.


mb1
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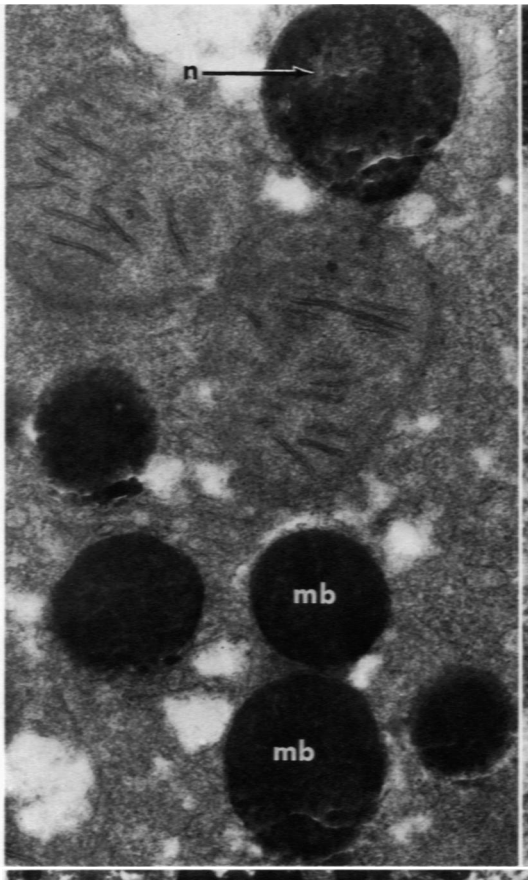
Figs 2 and 3. Normal rat liver, incubated for peroxidase at pH 8.0 for 30 min. Reaction product is seen in cellular organelles resembling microbodies (*mb*). Nucleoid (*n*) can be identified in some microbodies. Fig 2 × 70,000; Fig 3 × 120,000.

Fig 4. Male rat liver, 14 days afer CPIB treatment. Peroxidase reaction in sections incubated at pH 8.0 for 30 min. Numerous microbodies (*mb*) appear uniformly dense due to presence of reaction product throughout. Nucleus (*n*). Lead stain. × 8800.



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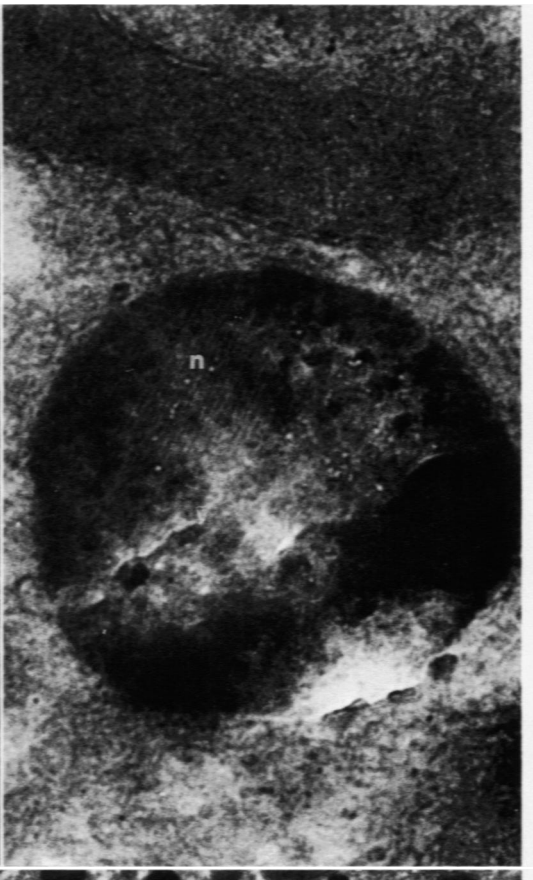

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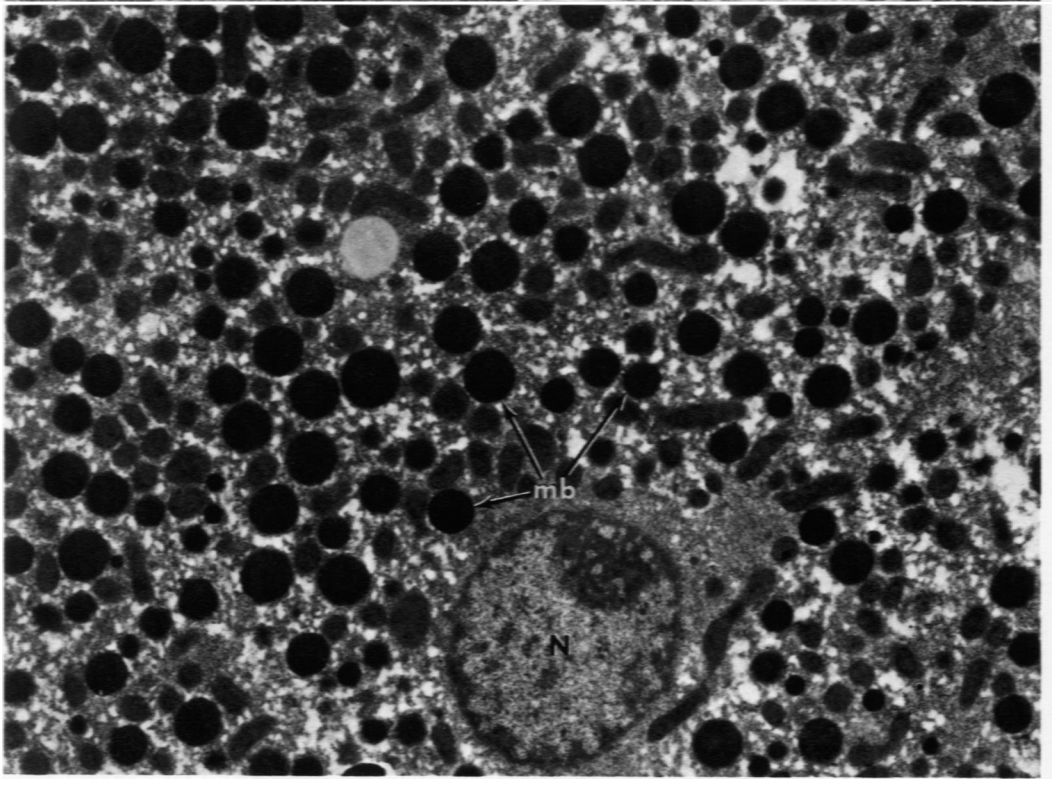

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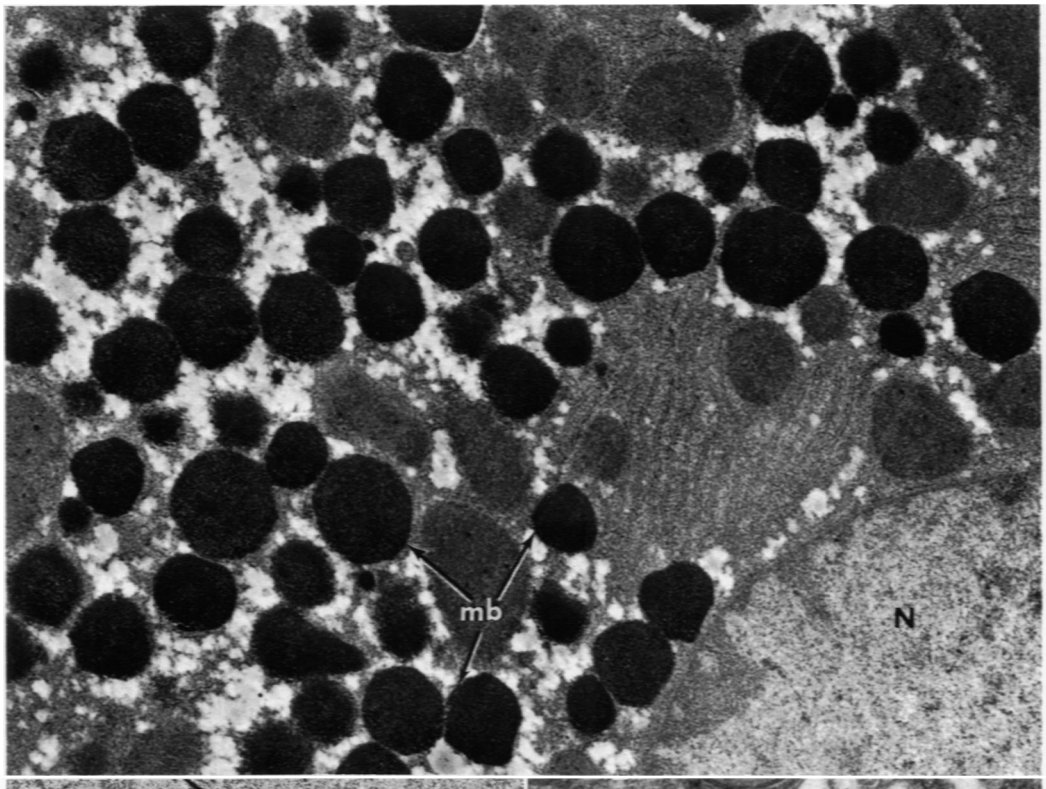
Fig 5. Peroxidase reaction in male rat liver treated with CPIB for 30 days. Sections were incubated at pH 7.6 for 60 min, postfixed in OsO_4. Reaction product in microbodies (*mb*) is intense. Nucleus (*n*). × 33,000.

Figs 6 and 7. Rat liver; CPIB for 36 days. Incubated for peroxidase at pH 8.0 for 60 min. Reaction product is visible in the cristae (*arrows*) of some mitochondria and in microbodies (*mb*). Lead stain. Fig 6 × 35,000; Fig 7 × 58,000.



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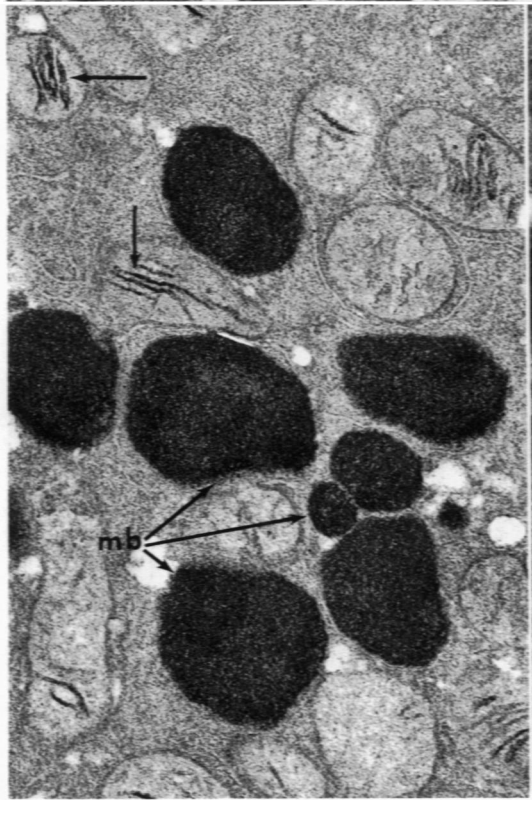

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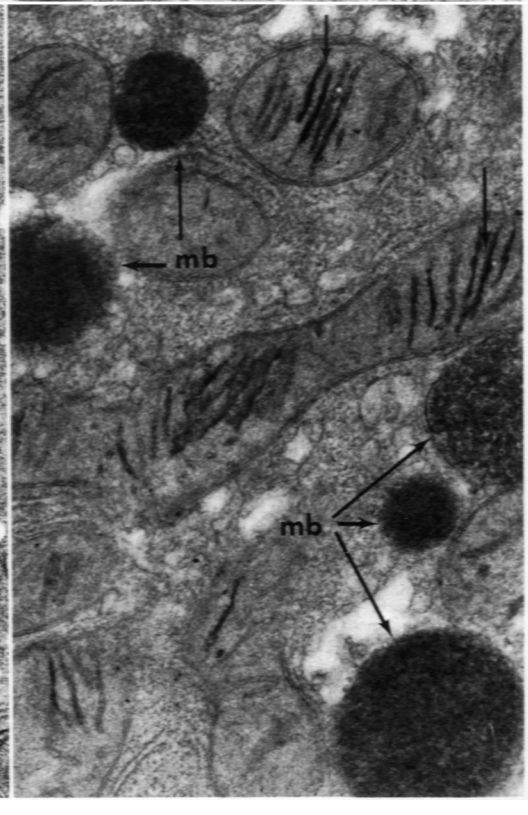

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Fig 8. Acatalasemic male mouse liver; CPIB for 18 days, incubated for peroxidase at pH 8.0 for 30 min. Increase in peroxidase positive organelles is conspicuous. Lead stain. × 4800.

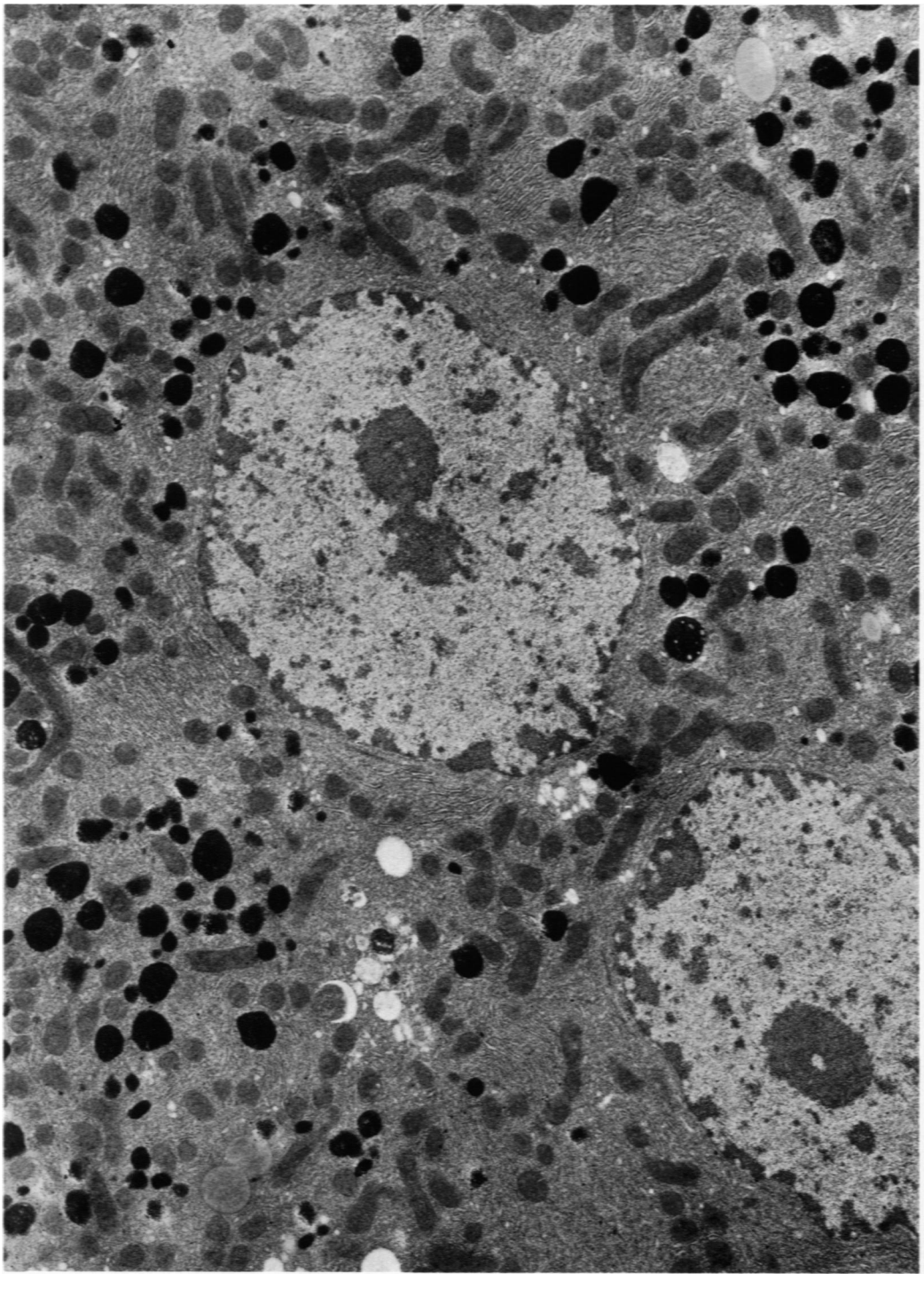

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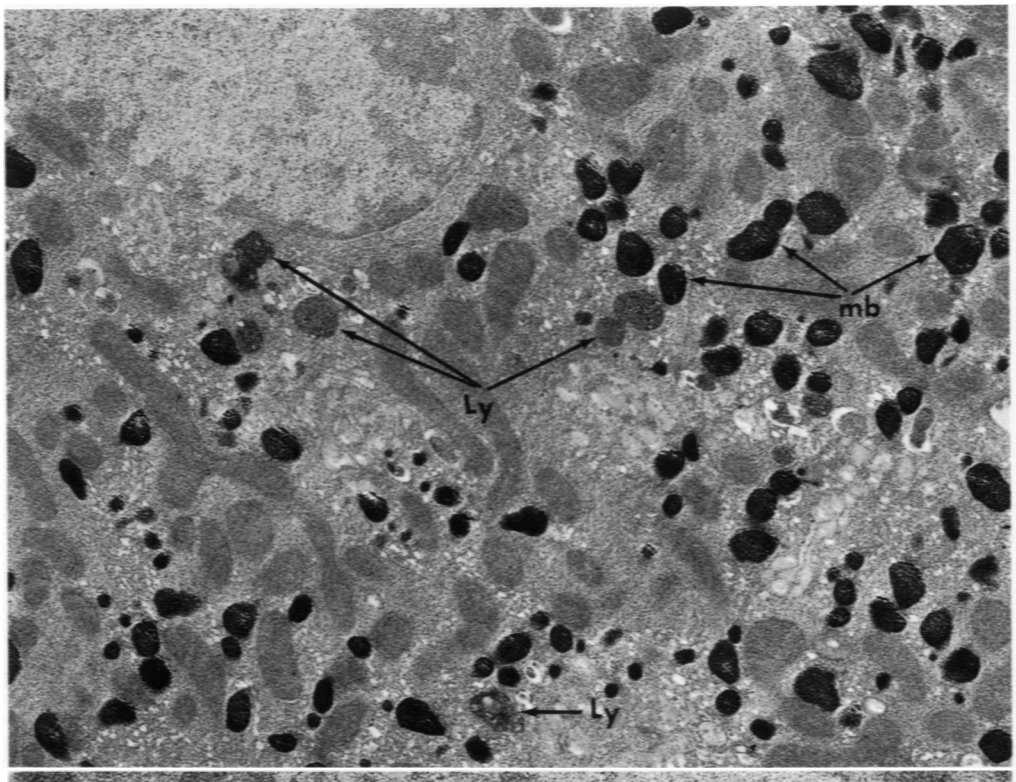
Fig 9. Acatalasemic female mouse liver; CPIB for 18 days, incubated for peroxidase at pH 8.0 for 30 min. Numerous microbodies (*mb*) show intense reaction product, while some lysosomes (*Ly*) show a faint mottling. × 11,000.

Fig 10. Control acatalasemic female mouse liver incubated for peroxidase. Peroxidase reaction is seen in an occasional microbody (*mb*). × 18,000.



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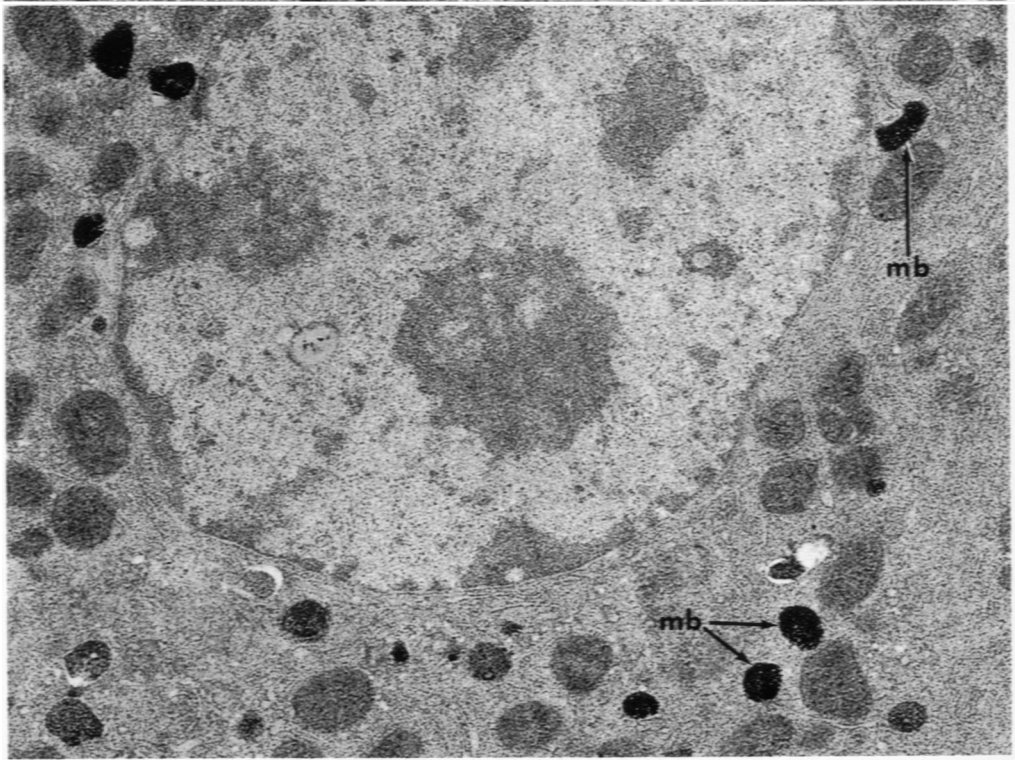

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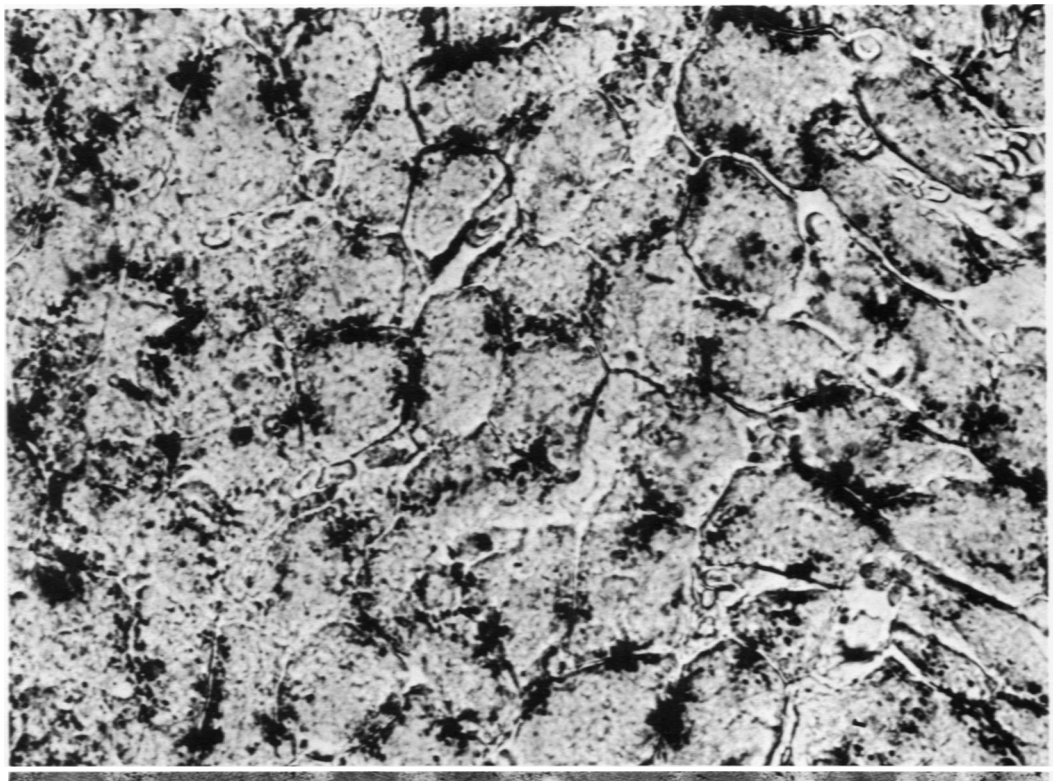
Fig 11. Acid phosphatase activity in male rat liver; CPIB for 3 weeks. Note typical peribiliary distribution of reaction product in liver cells. × 950.

Fig 12. Acid phosphatase; male rat liver treated with CPIB for 3 weeks. Reaction product is seen in a lysosome (*Ly*). Several anucleoid dense bodies labeled as microbodies (*mb*) do not show any reaction product. Nucleoids (*n*) are seen in some typical microbodies. × 66,000.



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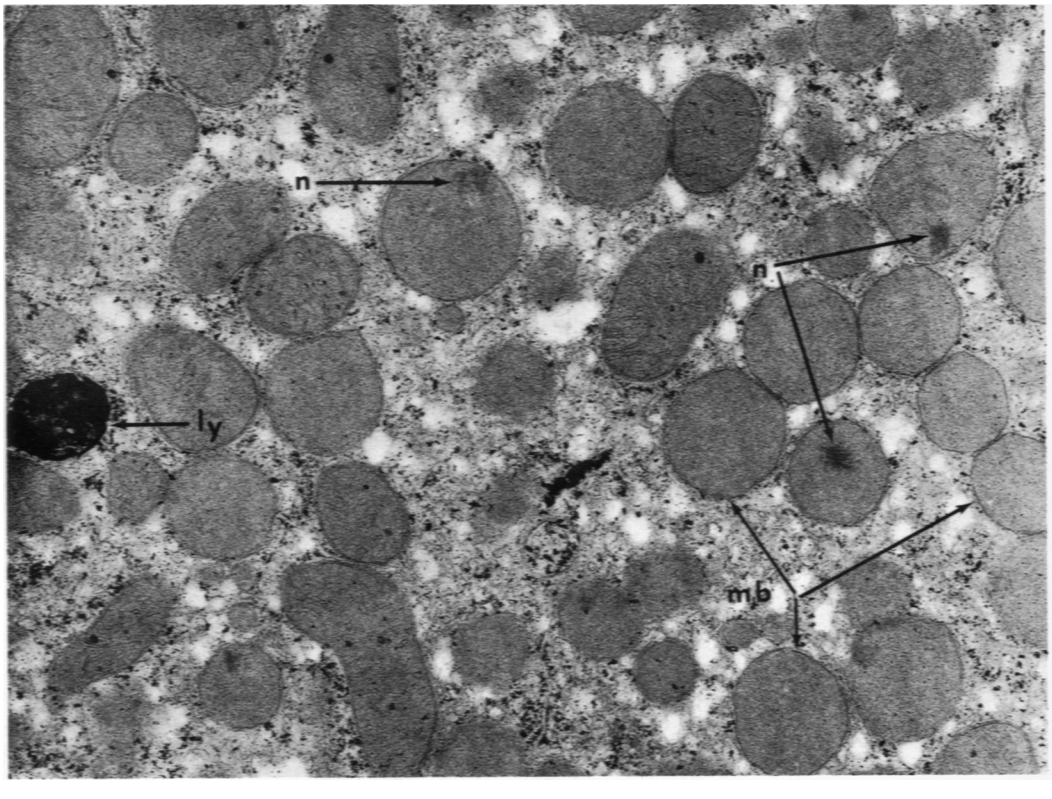

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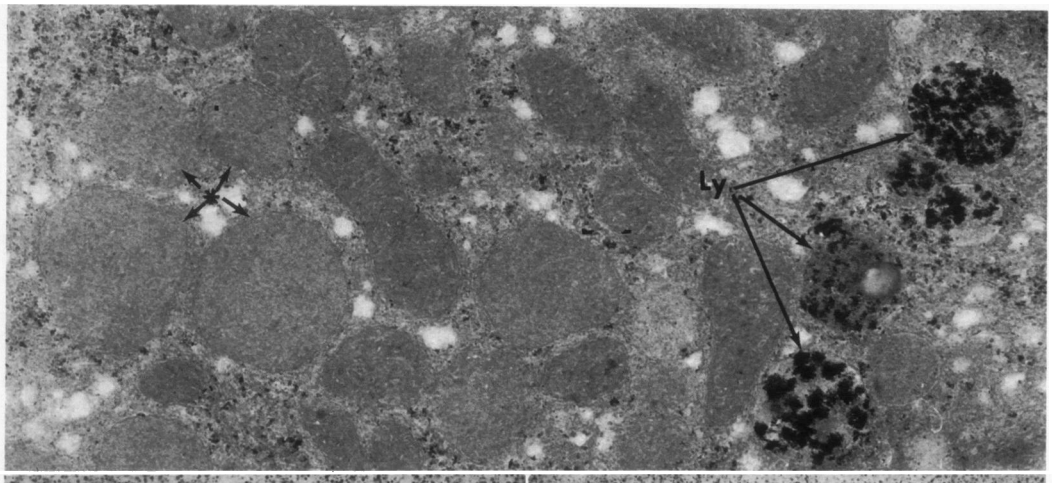

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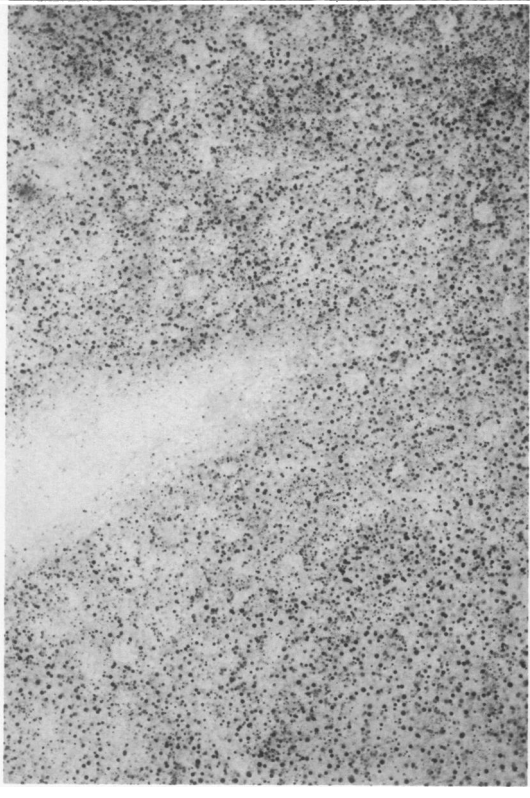

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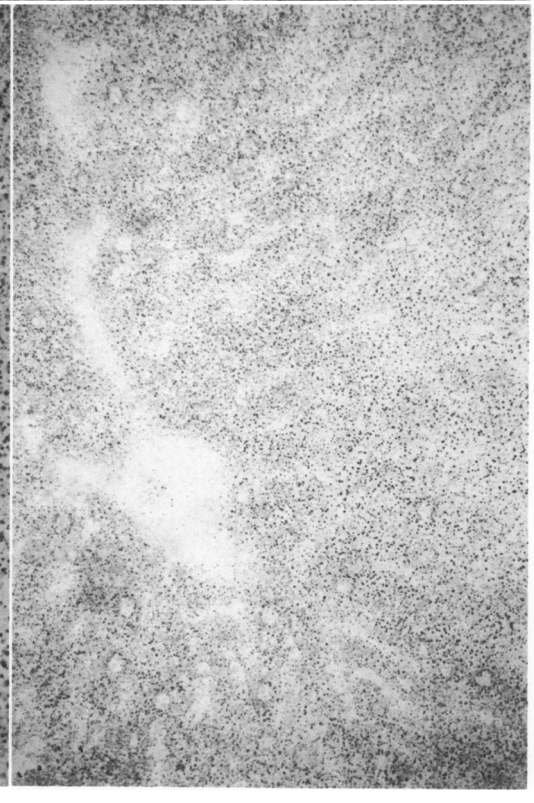

Fig 13. Acid phosphatase activity in male rat liver; CPIB for 6 weeks. Reaction product is seen in some lysosomes (*Ly*) while many round, single-membrane-bound structures (*) lacking the microbody nucleoid are acid phosphatase-negative. × 77,000.

Fig 14. Uricase activity, in male rat liver treated with CPIB for 30 days, is seen as dark granules dispersed throughout cytoplasm of parenchymal cells, differing markedly from distribution of acid phosphatase activity (see Fig 11). × 450.

Fig 15. Uricase activity in male acatalasemic mouse liver; CPIB for 36 days. Numerous dark granules in cytoplasm represent reaction product. × 250.