Microbodies in Experimentally Altered Cells

IX. The Fate of Microbodies

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Male rats were given 0.25% CPIB (ethyl chlorophenoxyisobutyrate) for 4 weeks at which time their liver cells showed the typical increase in number of microbodies (peroxisomes). During the first week after withdrawal of CPIB and simultaneous injection of allylisopropylacetamide (AIA), an inhibitor of catalase synthesis, the microbody matrix, in peroxidase preparations, showed marked decrease in electron opacity. In liver cells of rats from which CPIB was withdrawn but which were not given AIA, there were interruptions in the limiting membrane and almost total loss of microbody matrix within 3 days. Simultaneous with the loss of matrix content, the hepatic catalase activity decreased from an average 80 units/mg protein to less than 10 units/mg protein. In liver cells of *Mastomys natalensis*, a multimammate rodent, withdrawal of CPIB was associated with the frequent occurrence of bare microbody nucleoids situated free in the hyaloplasm. The ultrastructural observations and the associated decrease in catalase activity suggest that one means of disposal of microbodies is by rather rapid dissolution or leakage of their matrix enzymes into the surrounding hyaloplasm (Am J Pathol 67:541–554, 1972).

SEVERAL PREVIOUS STUDIES have dealt with factors that influence the proliferation of microbodies (peroxisomes) and increase in hepatic catalase activity following administration of a hypolipidemic drug, Clofibrate (CPIB, ethyl-α-p-chlorophenoxyisobutyrate).[1-5] Using immunochemical methods, Reddy *et al*[6] recently showed that, in male rats, CPIB enhances the rate of synthesis of hepatic catalase by approximately 80% in 3 days and maintains this enhanced synthesis throughout the duration of its administration. The biochemical studies of Poole *et al*[7,8] suggest that microbody proteins form a single pool and exchange material continuously or intermittently. The possibility of such exchange is given substantial morphological verification by the recent demonstration, in livers of acatalasemic mice, of continuities or channels between two or more adjacent microbodies.[9]

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Compared to studies on the biogenesis of microbodies, relatively little attention has been given to the mechanism of removal or disposal of microbodies either in normal animals or in rats previously given CPIB. In an earlier study,[2] it was noted that rat liver cells revert to normal with respect to their number of microbodies between 2 and 3 weeks after withdrawal of CPIB. One mechanism for removing microbodies was their incorporation into lysosomes, but this phenomenon appeared to be too infrequent to account for disposal of the large number of microbodies that must occur during the first 2 weeks after withdrawal of CPIB. Chiga *et al*[10] found that in CPIB-treated and normal rats given allylisopropylacetamide (AIA), an inhibitor of catalase synthesis, the first order rate constants of catalase degradation were 0.0276 and 0.0253, respectively. Accordingly, CPIB and AIA were used in the present experiments to determine whether there was additional ultrastructural evidence for alternative mechanisms of removal or disappearance of microbodies from rat liver cells.

Materials and Methods

Male F–344 rats weighing between 80 and 120 g were divided into 6 groups. Groups 1 through 4 were given 0.25% CPIB (Ayerst Laboratories, New York) in powdered chow diet for 4 weeks and treated as follows at that time: Group 1 remained on CPIB for an additional week; Group 2 was taken off CPIB and one animal was killed on days 1, 2, 3, 5, 7, 10, 12 and 14; Group 3 was taken off CPIB, given AIA and one animal was killed on days 1, 2, 3, 5 and 7. Group 4 remained on CPIB and was given AIA for 1 week. Group 5 served as untreated controls and Group 6 was given AIA only for 1 week. AIA was given twice daily by intraperitoneal injection at a dose of 200 mg/kg. A similar schedule of treatment and sacrifice was used for groups of *Mastomys natalensis*, a multimammate rodent. This species was added because we had previously observed occasional microbody nucleoids lying free in the hyaloplasm after treatment with CPIB;[11] this finding suggested previous dissolution of the remainder of the microbody.

Because the nucleoid is composed of uricase,[12] assay of hepatic uricase activity was done in *Mastomys* livers according to the method of London and Hudson [13] to determine whether the presence of free nucleoids was associated with a change in measured uricase activity.

For demonstration and localization of peroxidase activity, livers were perfused via the portal vein with 3% distilled glutaraldehyde in 0.15 M sodium cacodylate buffer, pH 7.4, and small pieces were fixed for 4 hours in the same solution. After fixation, the tissues were rinsed overnight in 0.1 M cacodylate buffer containing 0.22 M sucrose. Frozen sections, 40μ thick, were incubated at 37 C for 30 to 60 minutes at pH 9.0 in the diaminobenzidine (Sigma Chemical Co, St Louis, Mo) reaction medium of Novikoff and Goldfischer [14] modified from Graham and Karnovsky.[15] Controls consisted of incubations in which 0.02 M 3-amino-1, 2, 4-triazole (K and K Laboratories, Plainview, NY) 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 tetroxide and processed for electron microscopy. Acid phosphatase studies were performed on livers fixed for 4 hours at 0–4 C in 4.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4,[16] and in

incubation medium according to Gomori[17] with β-glycerophosphate and lead nitrate at pH 5.0. Controls consisted of incubation of sections in medium lacking glycerophosphate.

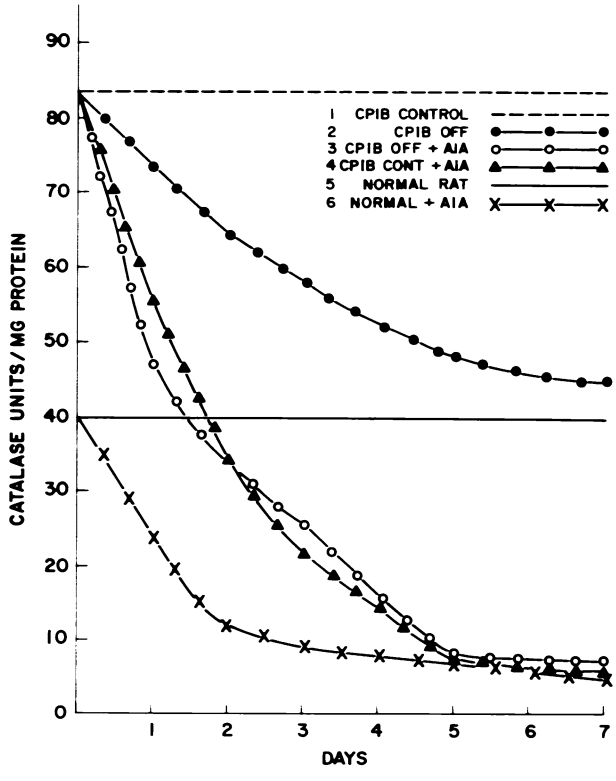
For determination of catalase activity, 1 g of liver in 4 ml of M/150 phosphate buffer was homogenized at 1–4 C with 20 strokes in a Potter-Elvehjem homogenizer at 400 rpm. Catalase activity was measured by the spectrophotometric method of Lück.[18] Total liver proteins were determined by the Lowry method.[19]

For electron microscopy, blocks of liver were postfixed in 2% osmium tetroxide buffered to pH 7.4 with s-collidine (2, 4, 6-trimethylpyridine; Vaughn, Inc, Memphis, Tenn), dehydrated in alcohol and embedded in epoxy resins. Some sections were stained with lead hydroxide.[20] Blocks of tissue prepared for ultrastructural cytochemistry were examined unstained.

Results

Biochemical

The hepatic catalase activity in all groups of rats is depicted in Text-figure 1. Animals that continued on CPIB for 1 week beyond the



TEXT-FIG 1—Catalase activity in rat liver. Day 0 represents the beginning of the experiment after Groups 1–4 had received CPIB for 4 weeks.

initial 4 week period (Group 1) maintained elevated catalase activity while untreated controls (Group 5) maintained the normal steady state level. Rats taken off CPIB but not given AIA (Group 2) showed a rapid decrease to normal range in hepatic catalase activity over a period of 7 days. All animals given AIA (Groups 3, 4 and 6) showed a similar rate of fall in hepatic catalase activity to approximately 5 units/mg protein over a period of 5 to 7 days. There was no significant difference between animals taken off CPIB at 4 weeks and given AIA (Group 3) and those that continued to receive CPIB during the week of AIA injection (Group 4). The results of assay of hepatic catalase activity in *Mastomys* were similar to those in rats except that, in animals taken off CPIB after 4 weeks, the activity remained significantly elevated during the following week. The average hepatic catalase activity in *Mastomys* that received CPIB for 4 weeks was approximately 80 units/mg protein. One week after withdrawal of CPIB from this species, the average activity was 65 units/mg protein compared to a control value, in this species, of 51 units/mg protein. Hepatic uricase activity in *Mastomys* did not change after administration or withdrawal of CPIB, but remained stable at approximately 6 units/mg protein.

Morphologic

Cytochemical studies indicated that there was no increase in number or size of lysosomes in any animals. Peroxidase preparations showed a decrease in electron opacity of the microbody matrix in the group of animals given AIA and withdrawn from CPIB (Group 3) (Figures 1 and 2). Otherwise, AIA did not appear to affect microbody ultrastructure but livers of AIA-treated animals showed proliferation of smooth endoplasmic reticulum as previously reported by others.[21,22] Peroxidase activity was detected occasionally within autophagic vacuoles (Figure 3).

Animals maintained on CPIB beyond 4 weeks (Group 1) showed typical increase in number of microbodies throughout the liver lobules as described previously.[1,2] Untreated controls (Group 5) had normal ultrastructure of the liver.

The principal alterations in microbody ultrastructure occurred in those rats from whom CPIB was withdrawn (Groups 2 and 3). During the first week after withdrawal of CPIB, several microbodies showed a marked decrease in electron opacity of their matrix even though, in the same field, other microbodies appeared normal (Figure 4). In most instances, there appeared to be almost total loss of matrix con-

tent with only the crystalline nucleoid remaining (Figures 5–10). Along with apparent loss of matrix, there were one or more interruptions in, or segmental loss of the limiting membrane (Figures 7, 8). In *Mastomys natalensis* the most conspicuous changes in microbodies occurred between 1 and 5 days after withdrawal of CPIB without injection of AIA. The changes were essentially similar to those that were present in rats. Microbodies in a single field varied from normal matrix density to almost complete loss of matrix content but with retention of the characteristic nucleoid. Nucleoids situated free in the hyaloplasm and not associated with microbodies or other organelles were common after withdrawal of CPIB (Figures 11, 12). The free nucleoids were identifiable as such because of their characteristic tubular crystalline structure, identical to that of nucleoids within intact microbodies of control *Mastomys*. Free nucleoids were also present in normal and CPIB-fed controls but appeared to be increased about twofold in animals taken off CPIB.

Discussion

In cell biology, considerable attention has been given to the morphogenesis of cell organelles, notably the membranes of the smooth endoplasmic reticulum, mitochrondria and microbodies. The reports of Essner [23] and Tsukada *et al* [24] suggest that in fetal livers of rats and mice the microbodies originate from specialized portions of the endoplasmic reticulum. Poole *et al* [7] hypothesized that newly synthesized microbody proteins move from the rough to the smooth endoplasmic reticulum and then accumulate in a dilatation of the latter. By this mechanism, it is suggested that the microbody contents are transferred directly from the ergastoplasmic system to the particulate organelle without involvement of the Golgi apparatus. Earlier studies on the incorporation of isotopically-labeled leucine into catalase of rat liver cell fractions provide supporting evidence for such a hypothesis.[25] Few data are available regarding the mechanism of decrease in number or disposal of microbodies. While the lysosome concept (sequestration of cytoplasmic organelles into autophagic vacuoles or foci of degradation and hydrolysis) explains adequately the removal of some organelles in both physiologic and pathologic conditions, it appears that such a mechanism is not the entire means for disposal of microbodies, since the incorporation of microbodies into lysosomes, which is identified by acid phosphatase positivity, is only rarely seen in livers of rats withdrawn from CPIB. Yet it is known from previous studies that after rats are given CPIB for 30 days and

then restored to normal diet, the excess number of hepatic microbodies and the elevated catalase activity are almost restored to normal between 7 and 14 days.[2] It was apparent that some additional mechanism for disposal of microbodies must exist. The present findings do not explain precisely the mechanism for removal of microbodies but do suggest, on morphological grounds, that these organelles are capable of gradual dissolution of matrix content and rupture of the limiting membrane leaving only the nucleoid in an electron-lucent remnant of the microbody. This gradual dissolution does not appear to require direct participation by other cell organelles, but instead, may be a property intrinsic to the microbody, possibly in conjunction with activity of the surrounding ground substance. It is quite probable that loss of matrix density is related to the decrease in catalase activity, since the two events occur together and catalase is one of the principal enzymes of the microbody matrix.[12] To what extent and in what sequence the remaining matrix enzymes are involved in the dissolution process could not be determined from the present study.

It appears that the mechanism(s) of catalase destruction and microbody disposal is the same in rats simply taken off CPIB as in those given AIA since the morphologic changes in microbodies were qualitatively identical in these groups.

Removal of microbodies in liver and kidney by the process of focal cytoplasmic degradation has been noted infrequently.[26] Microbodies have also been found in autophagic vacuoles of rat proximal tubular epithelium [27] and microbody nucleoids have been found in sites of focal cytoplasmic degradation in rat liver cells.[28] Hruban and Rechcigl have observed that, if this is the main type of removal of microbodies, they must break down rapidly after incorporation into the autophagic vacuoles. In plants, however, Vigil [29] observed that turnover of microbodies involved their incorporation into autophagic vacuoles as intact organelles which retain catalase activity. This apparent contradiction of evidence regarding the rate of destruction of microbodies and their catalase activity may be due to differences that exist between the mechanisms of microbody destruction in vertebrate liver and kidney and in castor bean endosperm. A mechanism for removing microbodies that bears close morphological resemblance to our findings occurs in the fat body of an insect, *Calpodes*. In this organ, during the fourth larval stage, microbodies undergo atrophy without isolation from the cell; the core or nucleoid is lost followed by the formation of myelin figures in the matrix.[30]

DeDuve and Baudhuin [12] postulated that peroxisomes grow contin-

uously for approximately 4 days and then burst, releasing their contents into the cytoplasm. More recently, Poole *et al*[8] suggested that all major protein components of the peroxisomes turn over with the same kinetics and that these organelles are destroyed as wholes by a random process. The present morphologic observations are consistent with these hypotheses except that the nucleoid, or core, which contains uricase, appears to be more impervious to destruction than the matrix, since in both rats and *Mastomys*, the nucleoid persists after the remainder of the microbody is almost completely destroyed. In a previous study,[2] we were unable to detect changes in rat hepatic uricase activity during CPIB feeding or after its withdrawal.

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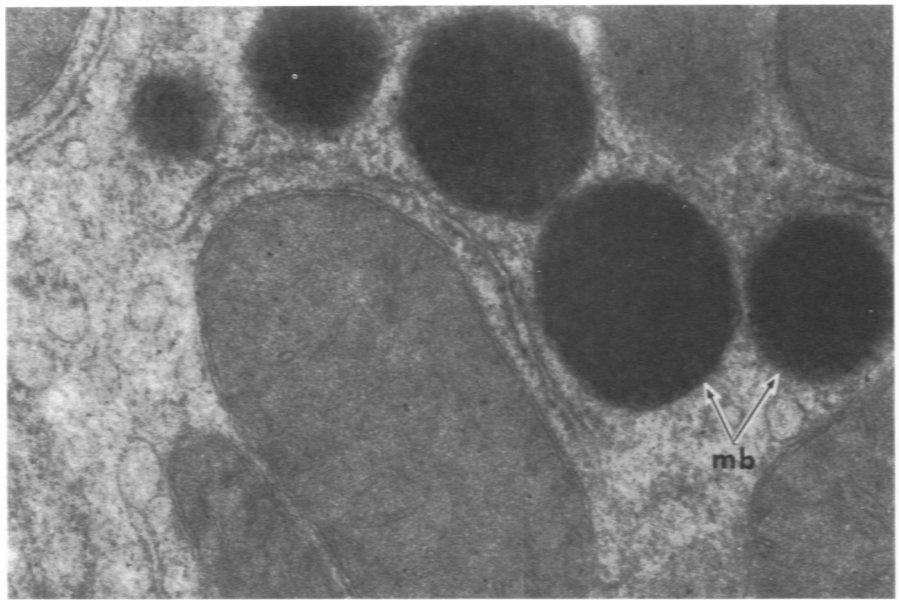
[*Illustrations follow*]

Legends for figures

Fig 1—Control rat liver incubated for peroxidase activity. The microbodies (*mb*) are very electron-dense (unstained, × 66,000).

Fig 2—Peroxidase preparation of rat liver 3 days after withdrawal of CPIB. Incubation was the same as used for Figure 1. The matrix of the microbodies (*mb*) is less electron-dense than in controls (unstained, × 66,000).

Fig 3—Peroxidase preparation of rat liver 3 days after withdrawal of CPIB. In addition to peroxidase activity in microbodies (*mb*) there is irregular peroxidase positivity in an autophagic vacuole (*unmarked arrow*) (unstained, × 66,000).



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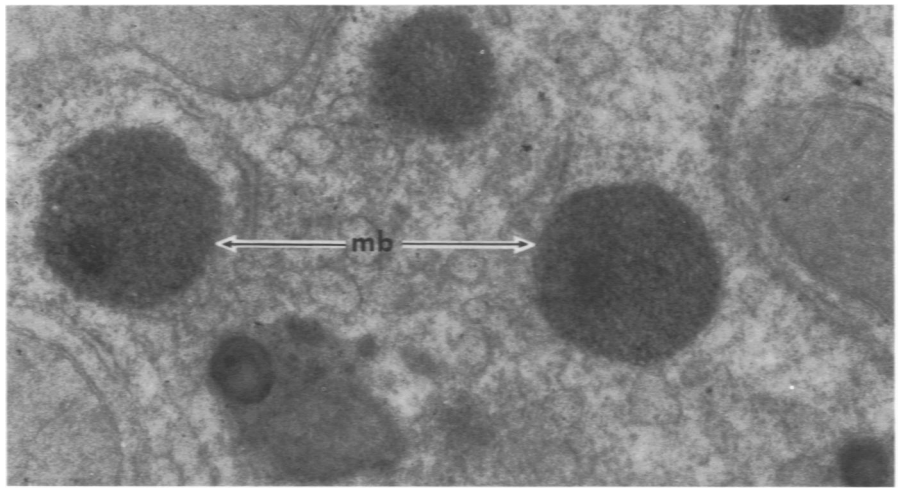

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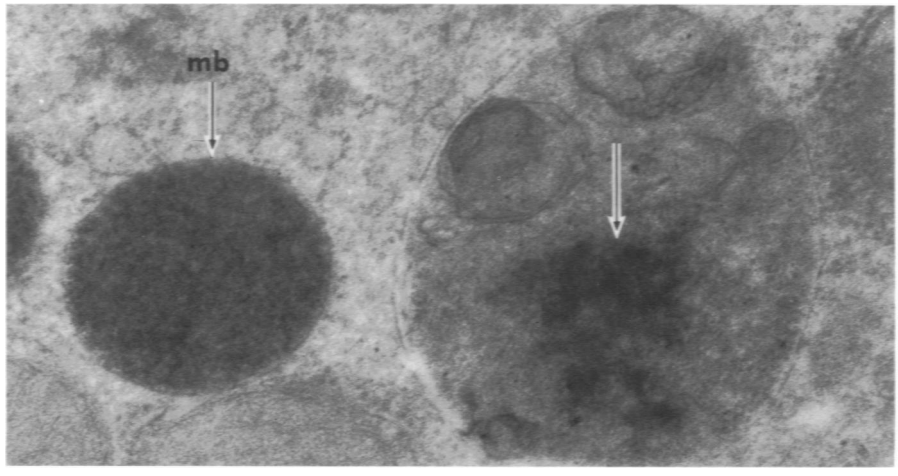

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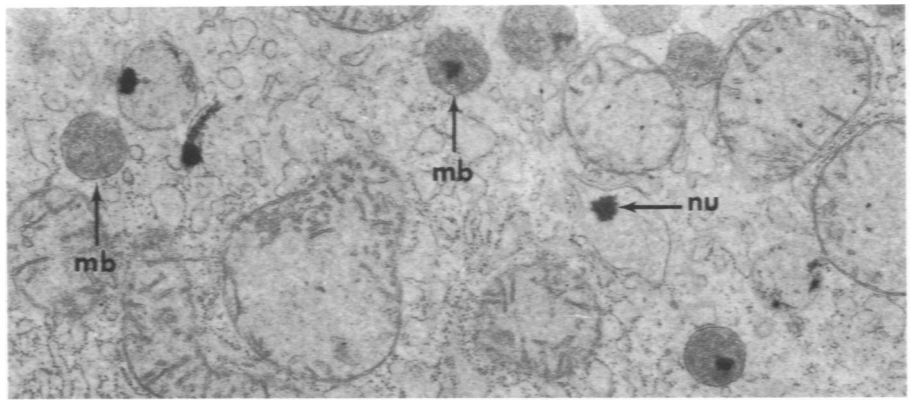
Fig 4—Rat liver 2 days after withdrawal of CPIB. In addition to microbodies (*mb*) with normal matrix density, there is one with an electron-lucent matrix identifiable as a microbody by the presence of the nucleoid (*nu*) (lead stained, × 19,200).

Fig 5–10—Rat liver 2 and 3 days after withdrawal of CPIB. These microbodies show loss or absence of matrix material indicated by marked electronlucency. Interruptions of the limiting membrane are apparent (*arrows*). Each microbody retains all or a portion of its crystalline nucleoid (lead stained, × 66,000).



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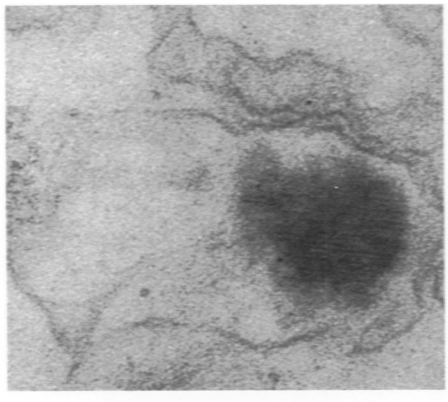

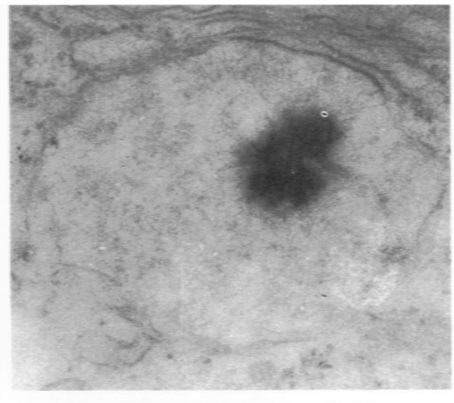

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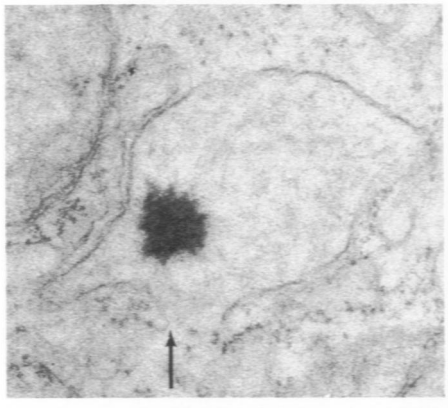

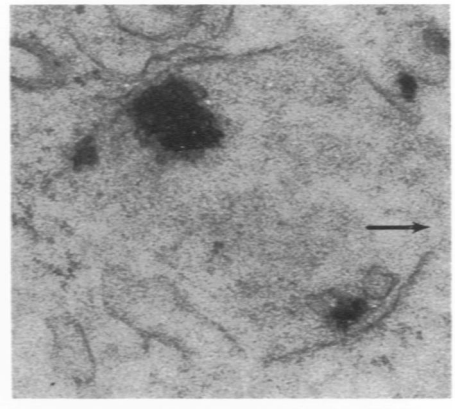

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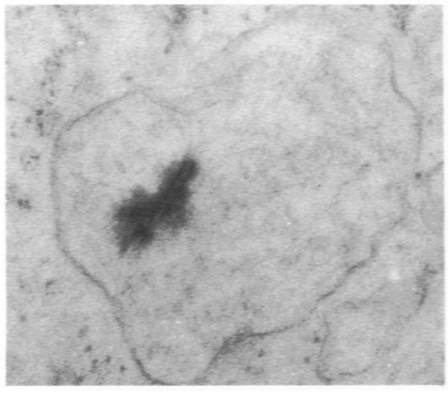

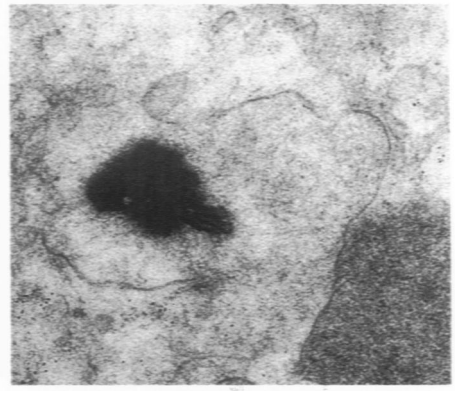

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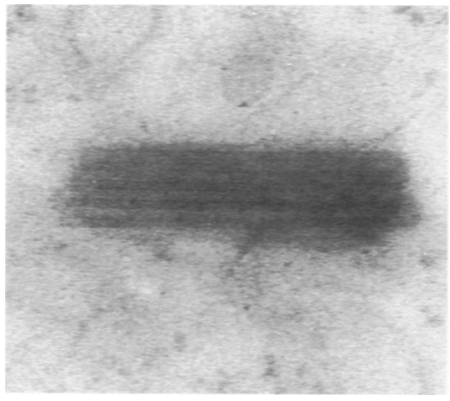

Fig 11—Mastomys liver 2 days after withdrawal of CPIB. A bare microbody nucleoid is situated free in the hyaloplasm (lead stained, × 118,000).

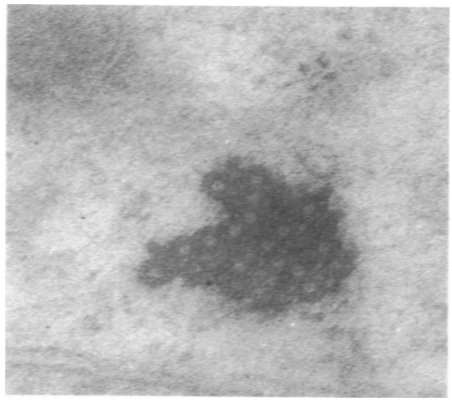

Fig 12—Mastomys liver 2 days after withdrawal of CPIB. A bare nucleoid in cross-section shows the typical crystalline lattice-work (lead stained, × 118,000).