NEW OBSERVATIONS ON MICROBODIES

A Cytochemical Study on CPIB-Treated Rat Liver

PETER G. LEGG and RICHARD L. WOOD

From the Department of Anatomy, University of Minnesota, Minneapolis, Minnesota 55455

ABSTRACT

The liver of male rats has been studied after CPIB stimulation by using the peroxidase reaction for localizing catalase in hepatic cells. CPIB administration leads to an increase in the number of microbodies, and it is suggested that one mechanism by which microbody proliferation occurs is a process of fragmentation or budding from preexisting microbodies. Reaction product was observed not only within the microbody matrix, but outside the limiting membrane of the microbody and in association with ribosomes of adjacent rough endoplasmic reticulum. This localization of reaction product is interpreted as evidence that catalase after synthesis on rough endoplasmic reticulum may accumulate near microbodies and may be transferred directly into these organelles without traversing the cisternae of the endoplasmic reticulum or Golgi apparatus.

INTRODUCTION

The existence of a distinct class of cytoplasmic organelle, the microbody or peroxisome, has been increasingly recognized since Rhodin's first description in 1954 (see reviews of de Duve and Baudhuin, 1966; Hruban and Rechcigl, 1969). The mechanism of formation of microbodies and their site of origin are poorly understood and controversial. Different cell organelles including smooth and rough endoplasmic reticulum, the Golgi apparatus, and multivesicular bodies have been implicated (5, 8, 9, 29, 40, 42, 43, 46, 47), but their definitive roles in microbody formation remain unclear.

Proliferation of microbodies in hepatic cells is known to occur in three main circumstances: (*1*) during fetal and early postnatal development (8, 9, 23, 32, 39, 48), (*2*) during the recovery phase following partial hepatectomy (36, 38), and (*3*) following the administration of certain chemical agents, notably salicylates and ethyl-α-*p*-chlorophenoxyisobutyrate (CPIB or Clofibrate, Ayerst Laboratories, New York) (16, 22, 30, 34, 40–42). Regular morphological observations on these systems have provided only limited information related to microbody development and have led to application of ancillary technics.

Microbodies are known to contain several oxidative enzymes which yield hydrogen peroxide as a reaction product and catalase, an enzyme capable of reducing hydrogen peroxide to water by two distinct mechanisms, catalatic and peroxidatic (7, 21, 24). The application of the peroxidase staining technic of Graham and Karnovsky (15), as modified by Novikoff and Goldfischer (28), has been successful in demonstrating microbodies at an ultrastructural level, presumably through the peroxidatic activity of catalase (4, 11–13, 44). Although the technic facilitates identification of microbodies, it has provided little additional insight into the mechanisms of microbody formation (4, 10, 11, 34).

The present study combines an additional modification of the cytochemical technic for demonstrating endogenous peroxidase activity with CPIB stimulation of microbody proliferation. The results suggest that (*a*) microbodies increase in number

through a fragmentation or budding phenomenon, (*b*) catalase after synthesis on rough endoplasmic reticulum may be inserted into microbodies without being transported through the cisternae of the rough endoplasmic reticulum, and (*c*) smooth endoplasmic reticulum and the Golgi apparatus are apparently not involved directly in microbody proliferation.

MATERIAL AND METHODS

Male Sprague-Dawley rats (Holtzman) 130–250 g in weight were used in this study. CPIB (Clofibrate, Ayerst)[1] was diluted with olive oil to provide 75 mg/0.5 ml and injected intraperitoneally in a dosage of 375 mg/kg daily. Liver was sampled at intervals from 12 hr to 5 days after the initial injection and examined by electron microscopy with conventional technics and after cytochemical reaction for peroxidase activity. Drug controls consisted of uninjected animals and animals injected with olive oil without CPIB. The regimen followed is summarized in Table I.

TABLE I

Experimental Regimen

	No. animals	Total No. inj./animal	Time of sacrifice after inital inj.
Experimental	3	1	12 hr
	3	1	18 hr
	5	1	24 hr
	4	2	2 days
	4	3	3 days
	3	5	5 days
Controls			
Olive oil alone	2	1	24 hr
Uninjected	5	—	—

Following these procedures, animals were anesthetized by intraperitoneal injection of sodium pentobarbital (Diabutal, Diamond Laboratories, Inc., Des Moines, Iowa), and the liver was perfused through the portal vein at room temperature with 3% glutaraldehyde containing 10 mM $CaCl_2$ and buffered to pH 7.4 with 0.1 M sodium cacodylate. Glutaraldehyde was obtained from Fisher Scientific Co. (Pittsburgh) and was vacuum distilled according to Fahimi and Drochmans (14), or the purified product was purchased directly from Ladd Research Industries, Burlington, Vermont. Perfusion was continued for 2–5 min after blanching of the liver, and then a portion was removed, placed on dental wax in fresh fixative, and diced into small cubes with razor blades. Fixation was continued at room temperature for a total time of 30 min.

Routine Preparations

Specimens were rinsed briefly in 0.1 M cacodylate buffer, pH 7.4, and postfixed in ice-cold 2.5% OsO_4 in the same buffer containing 5% sucrose. Subsequent dehydration in ethanol and embedding in Epon were routine (25). Sections cut with a diamond knife were stained with uranyl acetate (45) and lead citrate (35) and examined in a Siemens Elmiskop I electron microscope.

Cytochemical Preparations

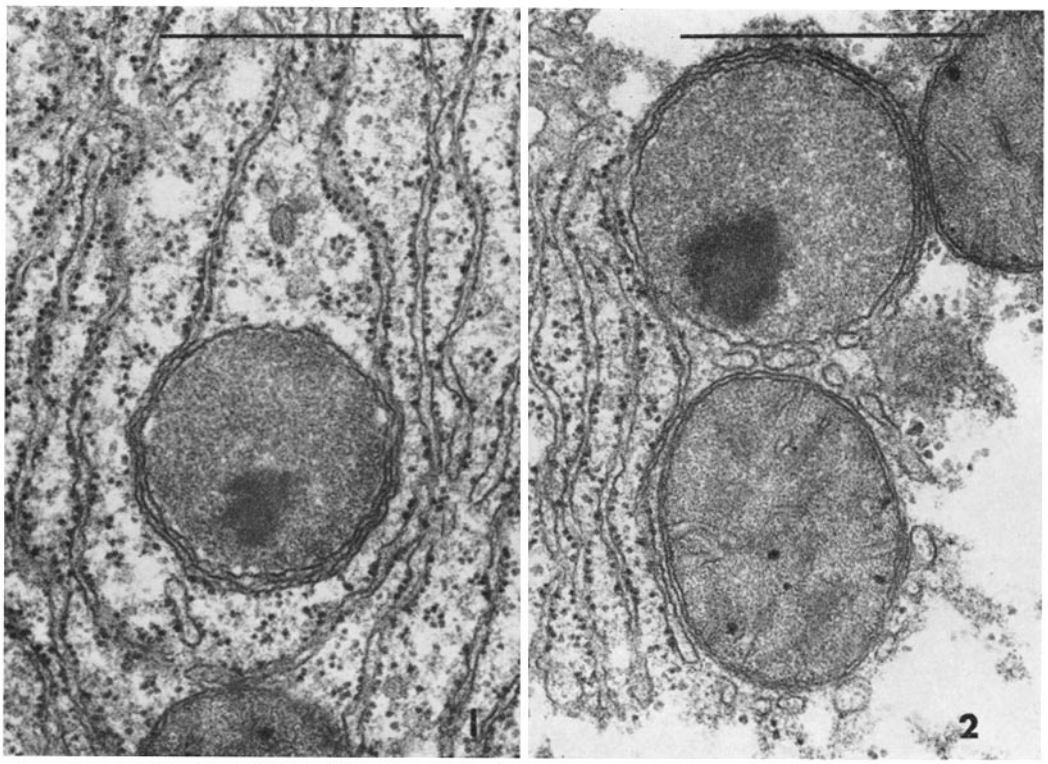
Glutaraldehyde-fixed tissue was rinsed several times in cold 0.1 M cacodylate buffer, pH 7.4, containing 5% sucrose, and selected specimens were chopped into 15- and 30-μ sections with a Sorvall TC-2 tissue sectioner (37). These sections were stored in the refrigerator overnight in fresh rinsing solution and stained the next day for peroxidase activity with a modification (48) of the procedure of Graham and Karnovsky (15). Sections were rinsed briefly in 0.05 M Tris-HCl buffer, pH 9, and placed for 1 min in 0.1% $CuSO_4$ dissolved in 0.9% NaCl (17). Following a brief rinse in Tris-HCl buffer, the tissues were incubated at room temperature for either 30 or 60 min in a medium composed of 10 ml of 0.05 M Tris-HCl, pH 9, 0.025 g of 3,3′-diaminobenzidine tetrahydrochloride (Sigma Chemical Co., St. Louis) and 0.1 ml of 3% H_2O_2. Cytochemical controls were run in the absence of H_2O_2 or with 0.02 M 3-amino-1,2,4-triazole (K & K Laboratories, Plainview, N.Y.) added to the complete medium (12). The 30-μ sections were rinsed briefly in distilled water and postfixed, dehydrated, and embedded in Epon as described above. Most of the cytochemical material was examined in the electron microscope without subsequent staining with heavy metal salts. The 15-μ sections were mounted on glass slides in glycerol-jel for light microscopic study.

OBSERVATIONS

Light Microscopy

After the cytochemical reaction for detecting peroxidase activity, microbodies appeared in normal hepatic cells as small, dense, circular particles similar in size to peribiliary dense bodies but distributed evenly throughout the cytoplasm. No evidence of a positive reaction could be localized to other hepatic cell organelles, but red blood cells and the granules of leukocytes stained intensely. Following the administration of CPIB, an increase

[1] We thank Dr. Jerome Noble, Ayerst Laboratories, for supplying the CPIB.



Scale marker on all figures equals 1 μ.

FIGURES 1 and 2 Routine preparations of normal rat liver showing close apposition of endoplasmic reticulum and microbodies. Ribosomes are absent where the reticulum membrane is closely applied to the microbody membrane. Fig. 2 shows the same relationship of endoplasmic reticulum to a mitochondrion. Double stained. × 40,000.

in the number and heterogeneity of microbodies was detected at 24 hr, and by 48 hr the changes were marked. However, as in normal tissue, there was no evidence of a positive reaction in other organelles. In the cytochemical controls incubated without H_2O_2 the staining of red blood cells and leukocyte granules remained intense, but only a weak reaction was observed in microbodies. In tissues incubated with aminotriazole in the medium, the microbody reaction was totally abolished but activity in blood cells remained.

Electron Microscopy

ROUTINE PREPARATIONS: CPIB treatment caused an increase in the number of microbodies in hepatic cells clearly evident at 18 and 24 hr after administration of the drug, but more prominent after longer treatment. The amount of tubular and vesicular smooth endoplasmic reticulum increased and there appeared to be a concomitant decrease in the amount of rough endoplasmic reticulum. Continuity between the membranes of rough and smooth endoplasmic reticulum was common at the periphery of areas comprised mainly of smooth endoplasmic reticulum (Fig. 4). By 3 and 5 days, mitochondria appeared more pleomorphic and the number of intramitochondrial matrix granules had increased noticeably. The nucleus, Golgi zone, and peribiliary dense bodies appeared normal in all of the experimental material.

Microbodies in stimulated cells resembled those in normal cells and consisted of a moderately electron-opaque, finely granular matrix surrounded by a single smooth membrane; many microbodies possessed a distinct core or nucleoid. In normal cells and in hepatic cells under short periods of stimulation with CPIB, most micro-

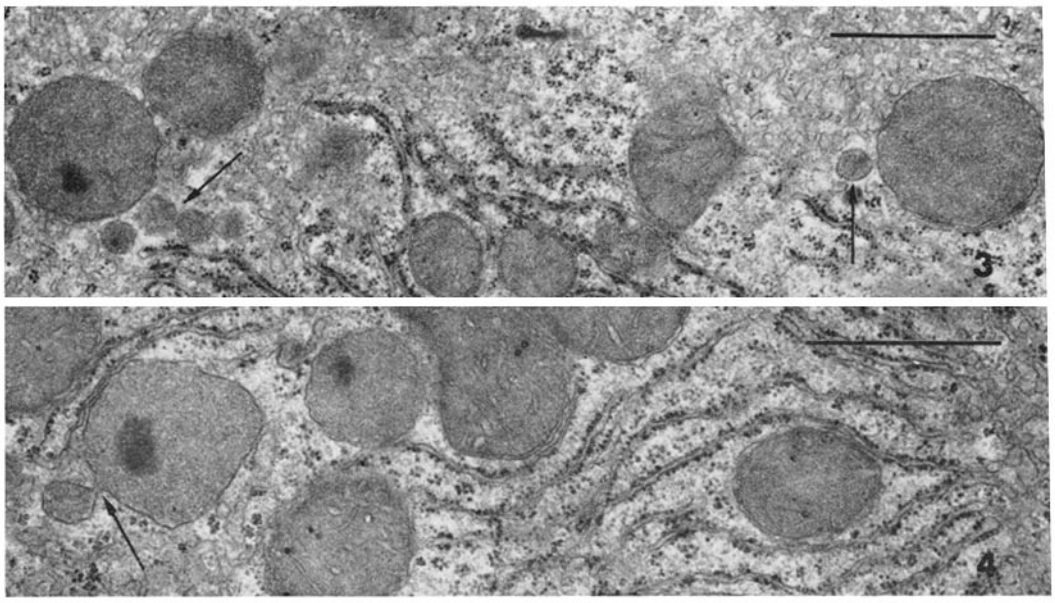


FIGURE 3 and 4 Routine preparations of rat liver 24 hr after CPIB treatment. Small vesicles (arrows) lie adjacent to microbodies. In Fig. 4, the small vesicle is attached to the larger microbody (arrow), and continuity of the rough and smooth endoplasmic reticulum is seen on the right. Double stained. Fig. 3, × 21,500; Fig. 4, × 25,500.

bodies were about 0.2–0.5 μ in size and circular or ovoid in outline, whereas at 2 days and later their size and shape varied widely.

A close spatial relationship between endoplasmic reticulum and microbodies was common. Many microbodies were situated between lamellae of rough endoplasmic reticulum, and occasionally a strand of rough endoplasmic reticulum almost completely encircled individual microbodies (Fig. 1). Where microbodies were closely enshrouded with endoplasmic reticulum, ribosomes were absent from the membrane surface closely applied to the microbody (Figs. 1, 2, 4). Tubules of smooth endoplasmic reticulum also often surrounded microbodies in a close spatial relationship. It was noteworthy that other particulate cell organelles such as mitochondria and lipid droplets were found in similar close relationships to both types of endoplasmic reticulum (Fig. 2). Despite careful examination, a continuity between the limiting membrane of the microbody and membranes of either rough or smooth endoplasmic reticulum was not demonstrated.

During the early phases (12–24 hr) of CPIB treatment, many microbodies showed small extensions of their limiting membrane. Smooth-walled vesicles were often closely associated with microbodies and, although these vesicles could not be positively identified as small microbodies, they contained material similar in appearance to that comprising the microbody matrix (Fig. 3). In some instances, it was possible to identify membrane continuity between the vesicle and the neighboring microbody (Fig. 4).

CYTOCHEMICAL PREPARATIONS: In tissue incubated in the complete medium, all microbodies showed a uniform intense reaction of the microbody matrix (Figs. 5, 6). The small surface extensions and small vesicles associated with the microbodies seen during the early phases of CPIB treatment also contained material which stained intensely (Figs. 5, 6). The connections between microbodies and the adjacent vesicles were more prominent in cytochemical preparations since these preparations were usually examined without further heavy metal contrasting (Figs. 6–8). In material sampled 3 and 5 days after commencing CPIB treatment, relatively few microbodies showed protrusions or connections with adjacent vesicles which by these times appeared larger than at earlier phases.

Microbody extensions were occasionally iden-

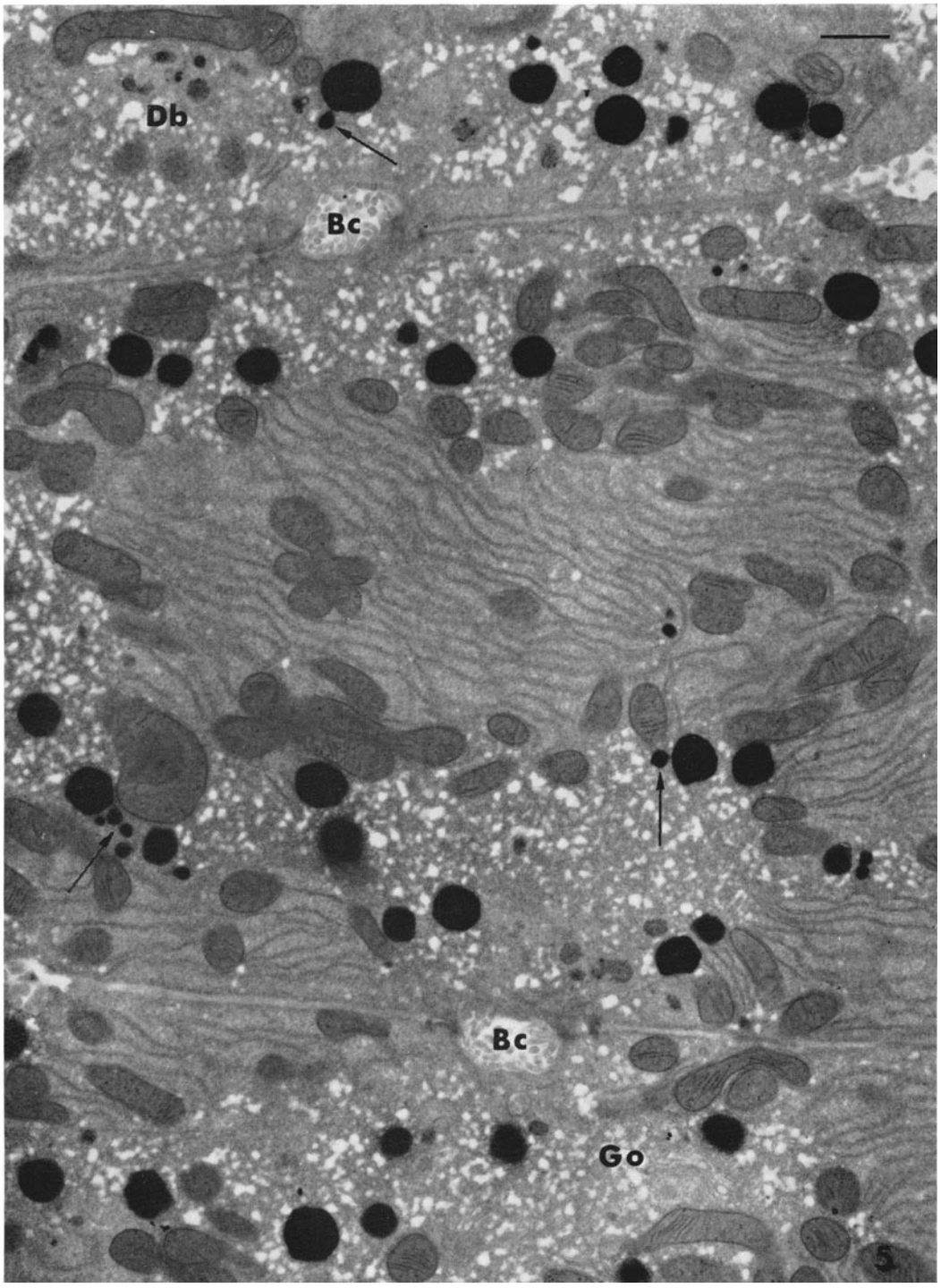


FIGURE 5 Peroxidase-stained rat liver 24 hr after CPIB treatment. There are numerous small peroxidase-positive vesicles (arrows), two of which appear connected to larger microbodies by delicate strands. Peribiliary dense bodies (*Db*) show some positive reaction, but no reaction occurs in the Golgi apparatus (*Go*). Bile canaliculus, *Bc*. No lead or uranyl staining. × 10,500.

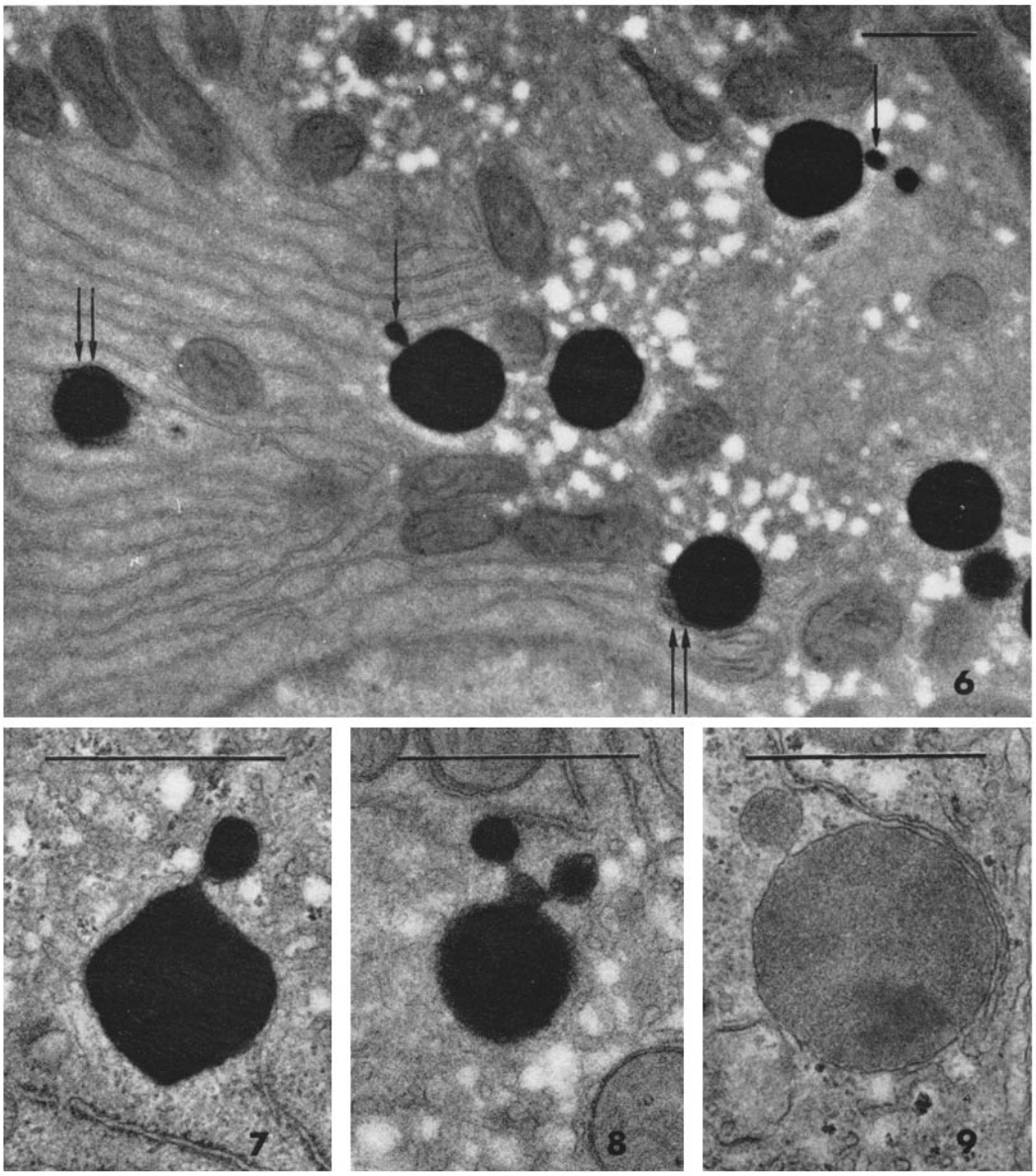
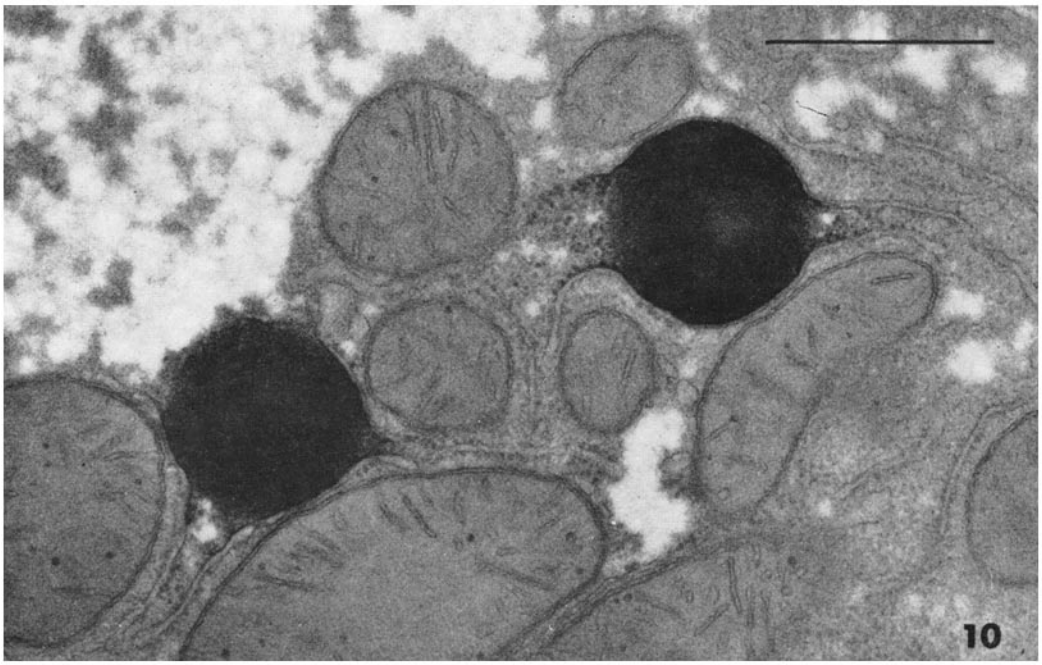


FIGURE 6 Peroxidase-stained rat liver 24 hr after CPIB treatment. Small vesicles associated with microbodies are indicated at the single arrows. Evidence of ribosomal staining appears at the double arrows. The reaction product is sharply localized around other microbodies in the micrograph. No lead or uranyl staining. × 16,000.

FIGURES 7–9 Rat liver 24 hr after CPIB treatment, showing microbodies in connection with small vesicles. Figs. 7 and 8 show material reacted for peroxidase whereas Fig. 9 is a cytochemical control run without H_2O_2. The preparations for Figs. 7 and 9 are double stained, and Fig. 8 shows material without lead or uranyl staining. × 32,000.



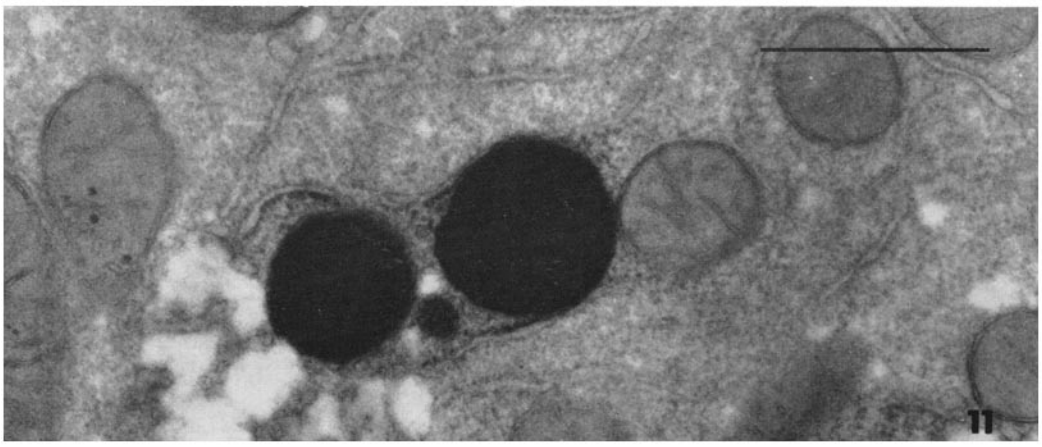


FIGURE 10 Normal rat liver reacted for peroxidase. Ribosomal staining is clearly evident. No reaction product is present in cisternae of the rough endoplasmic reticulum. No lead or uranyl staining. × 30,000.

FIGURE 11 Peroxidase-stained rat liver 18 hr after CPIB treatment. Ribosomal staining is similar to that in the normal liver (Fig. 10). No lead or uranyl staining. × 30,000.

tified in untreated normal hepatic cells reacted for peroxidase activity. The continuities between vesicles and microbodies also were seen clearly in CPIB-treated material used as cytochemical controls (Fig. 9).

A preferential distribution of microbodies was detected after the cytochemical reaction. In normal hepatic cells, microbodies appeared most commonly in areas of rough endoplasmic reticulum. During the early phases of CPIB stimulation (12–18 hr), they tended to be located at the boundary between rough endoplasmic reticulum and areas containing smooth endoplasmic reticulum or "glycogen." After 24 hr, the microbodies were more numerous within the areas of smooth endoplasmic reticulum or "glycogen."

An unusual distribution of staining was observed where microbodies were in close relationship to rough endoplasmic reticulum. Reaction product was present not only within the microbody matrix, but outside the limiting membrane of the microbody and in association with ribosomes of adjacent rough endoplasmic reticulum. This feature was most prominent at early intervals after CPIB administration (12–24 hr), but was also present in normal hepatic cells (Figs. 6, 10, 11). In contrast, those microbodies not closely related to rough endoplasmic reticulum but surrounded by tubules of smooth endoplasmic reticulum showed no reaction outside the microbody. At later phases of CPIB treatment, when microbody numbers were greatly increased (2–5 days), the ribosomal staining was markedly less evident than in earlier phases. Ribosomal staining was not eliminated by variations in fixation time, variations in incubation time including shorter periods, or by not utilizing preincubation in $CuSO_4$ solution.

Under all conditions used, microbodies with sharp localization of reaction product were present in the same cells in which the diffuse reaction was observed. Furthermore, there was no evidence of a diffuse staining related to other structures with a positive reaction for peroxidase, including red blood cells, the azurophil granules of leukocytes, and lysosomes.

At no stage was peroxidase activity demonstrated within cisternae of the rough or smooth endoplasmic reticulum or the Golgi apparatus of hepatic cells.

DISCUSSION

Conclusions concerning the origin of microbodies which have arisen from previous morphological studies (5, 8, 9, 29, 40, 42, 43, 46, 47) are based on three main factors: (*a*) alleged demonstration of continuity of membranes between the microbody and a precursor organelle, (*b*) similarity in electron opacity and texture of microbody contents and content of a precursor organelle, and (*c*) topographical relationships between the microbody and a precursor organelle. For each of these factors, the evidence on which it is based remains somewhat equivocal. First, there is little doubt that membranous extensions and protrusions of the limiting membrane of the microbody occur, but evidence that these are actually in continuity with endoplasmic reticulum is unconvincing. Second, similarity in electron opacity between material within the microbody and within adjacent organelles is not conclusive evidence that the two structures are related. The material observed within cisternae of the endoplasmic reticulum by many investigators may well represent totally unrelated substances. Third, proximity between microbodies and other organelles provides only inferential evidence of microbody origin.

The present study with a cytochemical technic for the localization of endogenous peroxidase activity was designed to explore microbody formation under conditions of rapid proliferation induced experimentally. The staining method involves oxidation of diaminobenzidine and deposition of a dense reaction product, the mechanism of which is not well understood. Nevertheless, many investigators have concluded that the deposition of reaction product in microbodies is due to the peroxidatic activity of catalase (4, 11–13, 44). It is possible that other unidentified peroxidases exist in microbodies and may be responsible for part of the observed staining, but use of this technic for studying microbody origin appears valid regardless of the absolute specificity of the reaction.

Though various mechanisms have been proposed for microbody proliferation, the possibility that new microbodies arise from preexisting microbodies has not been advanced. In the present study, small smooth-walled vesicles containing material similar in appearance to that comprising the microbody matrix were seen adjacent to microbodies, particularly during the early stages of CPIB treatment (12–24 hr). As already indicated, the morphological appearance of this material does not establish the vesicles as small microbodies, but the observation that this material possesses peroxidase activity suggests that the vesicles are indeed related to microbodies. Proliferation of microbodies is known to occur during CPIB administration (40–42), and the continuity seen between the membranes of the small vesicles and neighboring microbodies in the present study further suggests that these vesicles are derived, at least in some instances, from microbodies. Since other evidence concerning the fate of the small vesicles is lacking and since microbodies at later stages of CPIB treatment (2–5 days) are heterogeneous in size and shape, it seems reasonable to postulate that the small

smooth-walled vesicles are immature microbodies which have the capacity to increase in size and to form mature microbodies. In this scheme, the process by which the vesicles form could be considered either as fragmentation or as budding, but, whatever the exact mechanism, the end result would be the formation of new microbodies from preexisting microbodies.

Other evidence that catalase in newly formed microbodies may be derived from preexisting microbodies has recently been presented. Reddy et al. (34) found that when CPIB was administered to a strain of mice genetically deficient in catalase the number of peroxidase-positive microbodies increased but there was no measurable increase in liver catalase activity. In an attempt to account for this inconsistency, they suggested that a redistribution of catalase occurs; the means by which this might be accomplished was not elaborated. The process of microbody fragmentation or budding would readily explain their findings.

Aside from protrusions attributable to this phenomenon, the present observations show no evidence of microbody membrane continuity with other membranous organelles. It is possible that continuities do exist but, if so, they must be infrequent. This finding does not agree with the observations of Novikoff and Shin (29) and is directly opposed to the concept that microbodies are always attached to smooth endoplasmic reticulum in vivo. The continuities between microbodies and rough endoplasmic reticulum reported by Essner (9) in fetal mouse liver and by Tsukada et al. (43) in fetal rat liver were not confirmed in adult tissue. Different mechanisms may be involved in initial microbody formation as distinct from microbody proliferation in the adult, but it is noteworthy that such continuities were not observed in fetal rat liver by Wood (48).

It is of interest that in the present study no peroxidase reaction product was found within cisternae of the endoplasmic reticulum or Golgi apparatus. This observation has several possible explanations. The most obvious is that catalase is present at these sites but at a concentration below the sensitivity of the cytochemical technic (3, 4). Although this possibility cannot be refuted, the intensity of microbody staining and the fact that intracisternal staining of peroxidase has been observed in developing rat leukocytes with the same technic[2] suggest that the sensitivity of the technic may be sufficient to detect catalase at these sites. However, it must be noted that the ease of demonstration of peroxidase activity within cisternae of endoplasmic reticulum and Golgi apparatus of developing leukocytes is species dependent (1–3, 27, 49). Another explanation is that the enzyme is present in the endoplasmic reticulum in an inactive form, a tenable hypothesis but difficult to test. A third possibility is that catalase is not segregated into endoplasmic reticulum or Golgi cisternae after synthesis, but remains in the cytoplasmic matrix to be transferred directly into microbodies. The latter possibility deserves consideration in light of published biochemical evidence on catalase synthesis.

Biochemical studies by Higashi and Peters (18, 19) have shown that catalase is synthesized by membrane-associated ribosomes and transferred to particulate bodies, which can be assumed to be microbodies (their fractionation procedures did not separate microbodies from mitochondria). They found no evidence that the newly synthesized catalase is transferred into cisternae of rough or smooth endoplasmic reticulum, and they concluded tentatively that catalase either becomes soluble and migrates to microbodies or is transferred directly from the reticulum membrane to its definitive site.

In the present study, the ribosomal staining, the close apposition of strands of endoplasmic reticulum and microbodies, and the lack of peroxidase activity within the cisternae of endoplasmic reticulum are interpreted as morphological evidence that catalase, after synthesis on the ribosomes, may actually follow the pathways suggested by the biochemical studies of Higashi and and Peters (18, 19). The possibility that the ribosomal staining represents cytochemical diffusion artifact must be considered and cannot be totally discounted from the present evidence. Nevertheless, it is thought to be unlikely for the following reasons: (*a*) Ribosomal activity was not seen at all microbodies enwrapped with rough endoplasmic reticulum. (*b*) Under all conditions used, microbodies with sharp localization of reaction product were present in the same sections that showed microbodies with adjacent ribosomal staining. (*c*) Variations in fixation and staining procedures (shorter incubation times, lower con-

[2] Wood, R. L., unpublished data.

centrations of diaminobenzidine, no $CuSO_4$ treatment) altered the intensity of staining, but had no significant effect on localization of reaction product. (*d*) The effect was more pronounced in early stages of CPIB stimulation, whereas at later stages, when the number of microbodies had increased greatly, ribosomal staining was markedly less evident than at early stages. (*e*) There was no evidence of a diffuse reaction in relation to red blood cells, peribiliary dense bodies, or phagocytic vacuoles, all of which had peroxidatic activity.

The hypothesis that catalase may be synthesized at ribosomes and enter the microbody directly remains tentative, and confirmation obviously depends on further study. Such a route does not conform with the generally accepted tenets concerning synthesis and sequestration of proteins; if the hypothesis is to be entertained seriously, the mechanism has to be established by which catalase, a large molecular weight protein (mol wt 249,000 [33]), passes across the microbody membrane. There is no direct evidence from this study to indicate by what means this might be accomplished, but other studies provide some information relevant to the problem. First, it has been shown that the microbody membrane differs in osmotic and permeability properties from other cell membranes (6, 7). Secondly, there is biochemical evidence to indicate that smaller enzymatically active molecular forms of catalase exist in the hepatic cell (20, 26, 31). These lower molecular weight configurations could conceivably be the form of catalase at the time of incorporation into the microbody.

Thus, although additional knowledge is required before a definite conclusion can be reached, the possibility that catalase may be inserted directly into microbodies from ribosomes without involving transit through the endoplasmic reticulum or Golgi apparatus deserves consideration in spite of its being an unorthodox concept.

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