PEROXIDASE ACTIVITY IN RAT LIVER MICROBODIES AFTER AMINO–TRIAZOLE INHIBITION

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ABSTRACT

The in vivo effects of 3-amino-1,2,4-triazole (AT) on the fine structure of microbodies in hepatic cells of male rats has been studied by the peroxidase-staining technique. Within 1 hr of intraperitoneal injection AT abolishes microbody peroxidase-staining, and the return of staining coincides temporally with the known pattern of return of catalase activity following AT inhibition; this is further evidence that the peroxidase staining of microbodies is due to catalase activity. Peroxidase staining reappears in the microbody matrix without evidence of either massive degradation or rapid proliferation of the organelles. Furthermore, during the period of return of activity, ribosomal staining occurs adjacent to microbodies whose matrix shows little or no peroxidase staining. These observations are interpreted as evidence that (a) catalase is capable of entering preexisting microbodies without traversing the cisternae of the rough endoplasmic reticulum or the Golgi apparatus, and that (b) the ribosomal staining is probably not cytochemical diffusion artifact and may represent a localized site of synthesis or activation of catalase.

INTRODUCTION

Recent biochemical and morphological studies are providing new information on the biological significance of microbodies, or peroxisomes, in rodent hepatic cells, but many aspects remain uncertain. De Duve and his coworkers have demonstrated convincingly that these organelles contain the enzyme catalase in appreciable quantities (1, 4, 17). The association of catalase with hydrogen peroxide-forming enzymes and the known peroxidatic activity of catalase lead de Duve to propose the "peroxisome concept" of microbodies in which catalase plays a prominent role in the metabolism of hydrogen peroxide (4).

The fundamental importance of catalase in this proposed theory suggests that electron microscope studies with the peroxidase-staining technique of Graham and Karnovsky (9) may provide additional insight into the origins and functions of the microbody and its components. Recent studies in our laboratories (15) have used this approach in combination with drug treatment to stimulate microbody proliferation. These studies suggest that new microbodies may arise from preexisting microbodies and that catalase, after formation on the ribosomes of rough endoplasmic reticulum, may be transferred directly into microbodies without passing through the cisternae of either the endoplasmic reticulum or the Golgi apparatus. The validity of the latter concept is partly dependent on the significance of observed peroxidase staining associated with ribosomes adjacent to microbodies. This was interpreted tentatively as representing the synthetic site of catalase, but diffusion of reaction product from altered microbodies remains a distinct possibility.

There is biochemical evidence that catalase

within the hepatic cells of rats has a rapid turnover with an approximate half-life of 1½ days (4, 22, 24), and Poole et al. (22) recently suggested that this may represent the turnover of the whole microbody. At this time, there is little morphological evidence to support such a rapid turnover of hepatic microbodies as complete entities, although they occasionally appear within autophagic vacuoles.

Several investigators have shown that the biochemical activity of liver catalase may be inhibited by 3-amino-1,2,4-triazole (AT), a potent herbicide and defoliant, which produces a rapid decrease in liver catalase activity after injection into rats (10, 11). Later studies (8, 14, 19, 20, 22-24) indicate that AT combines irreversibly with the catalase and apparently destroys its enzymatic activity without affecting its synthesis, this feature being exploited for in vivo studies on catalase turnover (14, 22-24). Because of its inhibitory actions on catalase, AT has been used as cytochemical control for microbody staining with the peroxidase technique; it totally abolishes the microbody reaction on addition to the reaction medium (2, 6, 7, 13, 25, 26, 30).

The in vivo effects of AT on the fine structure of the hepatic cell and its contents, particularly microbodies, have not been reported in detail. With the peroxidase-staining technique, the present study examines the activity of catalase in rat liver at short time intervals after a single intraperitoneal injection of AT. The results are correlated with the available evidence on turnover of catalase and microbodies, and they support the concept that catalase may be transferred directly into microbodies without passing through the cisternae of the endoplasmic reticulum or Golgi apparatus.

MATERIALS AND METHODS

Male Sprague-Dawley rats, 130-200 g in weight, were housed in individual cages and given free access to food and water until the time of sacrifice. 3-amino-1,2,4-triazole (AT) (K. and K. Laboratories Inc., Plainview, N. Y.) was made up with distilled water to provide 100 mg/ml and injected intraperitoneally in a dosage of 1 g/kg. Three rats comprised each experimental group. Liver was sampled at intervals of 1, 2, 4, 6, and 8 hr after the AT injection. Material was examined by electron microscopy with conventional techniques and after the cytochemical reaction for peroxidase activity.

Initial Fixation

Animals were anesthetized by intraperitoneal injection of sodium pentobarbital (Diabutal, Diamond Laboratories, Des Moines, Ia.), 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 purchased from Ladd industries, Burlington, Vermont. Perfusion was continued for 2-5 min after blanching of the liver, and a portion was removed, placed on dental wax in fresh fixative, and sliced 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 sodium cacodylate buffered to pH 7.4 and postfixed for 1 hr in ice-cold 2.5% OsO_4 in the same buffer containing 5% sucrose. Subsequent dehydration in ethanol and embedding in Epon were routine (18). Sections cut with a diamond knife were stained with uranyl acetate (31) and lead citrate (27) for examination in a Siemens electron microscope.

Cytochemical Preparations

The technique used has been described in full in another report (15). Briefly, the glutaraldehyde-fixed tissue was rinsed in cacodylate buffer at pH 7.4 and cut into thick sections with a Sorvall TC-2 tissue sectioner (Sorvall, Ivan, Inc., Norwalk, Conn.) (28). The sections were stored overnight in a refrigerator in buffer and stained the next day with a modification (32) of the procedure of Graham and Karnovsky (9), the incubation time being 60 min for all tissues. Cytochemical controls were run in the absence of H_2O_2. Material for electron microscopy was postfixed, dehydrated, and embedded in Epon as described above, but usually was examined without subsequent staining by heavy metal salts. Parallel sections were mounted in glycerol-jel on glass slides for light microscopy.

OBSERVATIONS

Routine Preparations

The administration of AT did not seem to affect the general morphology of the hepatic cell in the first 8 hr after injection; the nucleus, both rough and smooth endoplasmic reticulum, mitochondria, and lysosomes appeared normal in all of the experimental material. It was not possible to detect
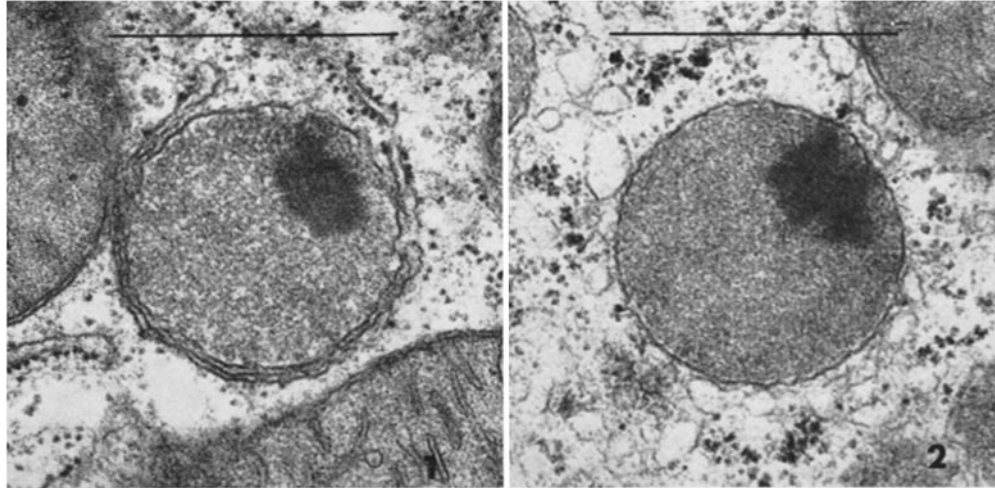
Marker on all figures is 1 μ.

FIGURE 1   Microbody from a routine preparation of normal rat liver, showing the uniform, finely granular matrix material and the prominent nucleoid. Double stained. × 38,000.

FIGURE 2   Microbody from a routine preparation of an animal injected with AT 4 hr prior to sacrifice. The limiting membrane, matrix material, and nucleoid are normal in appearance. The microbody matrix appears slightly more homogeneous than in Fig. 1, but this is caused by greater section thickness. Glycogen granules are present near the microbody. Double stained. × 38,000.

morphological differences between microbodies in untreated and treated animals (Figs. 1, 2). There was no obvious increase or decrease in the number of microbodies, and there was no indication that a massive destruction or rapid proliferation of microbodies was occurring. Microbodies maintained normal spatial relationships to other cell organelles, including the endoplasmic reticulum and the Golgi apparatus.

The limiting membrane of the microbody did not appear to be altered by the administration of AT, and well-formed membranous protrusions were seen rarely. As in normal hepatic cells, it was not possible to demonstrate continuity of the microbody membrane with other membranous organelles. At no stage after the injection of AT was there any evidence of abnormal accumulation of material within the cisternae of rough or smooth endoplasmic reticulum.

Cytochemical Preparations

With the light microscope, small dark brown granules were visible within the cytoplasm of untreated hepatic cells against a diffuse red-brown background. These granules, presumably mostly microbodies, were not present in controls lacking H_2O_2 in the incubation medium. They were not visible in material treated with AT until 8 hr after the injection when pale brown granules were just discernible. The background stain was not noticeably altered by AT administration, and red blood cells stained intensely in both normal and experimental liver tissue.

At the electron microscope level, all microbodies in normal hepatic cells incubated in the complete medium showed a uniform, intense reaction in their matrix (Fig. 3). Most microbodies possessed reaction product clearly localized to the matrix alone, but, in some instances, staining occurred outside the microbody-limiting membrane in association with ribosomes and membranes of adjacent strands of rough endoplasmic reticulum (Fig. 7). Ribosomal staining appeared occasionally at distances up to 1 μ from microbodies without evidence of intermediate accumulation of reaction product.

Red blood cells, azurophil granules of leukocytes, lysosomes in Kupffer cells, and peribiliary dense bodies all showed endogenous peroxidase activity, but there was no evidence of a diffuse reaction related to these structures. Sporadic staining of mitochondrial cristae occurred also.

At 1 hr following AT administration there was no evidence of peroxidase activity in the micro-
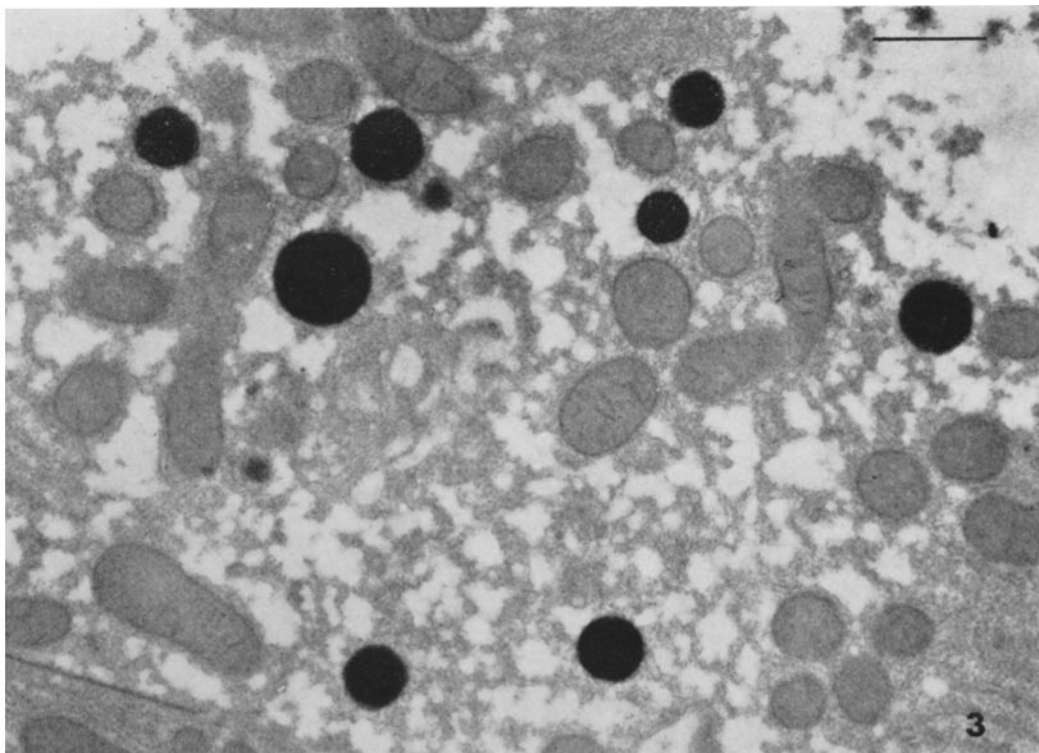
FIGURE 3 Normal rat liver reacted for peroxidase activity, showing prominent microbody staining. The nucleoid is masked by the strong peroxidase reaction. No uranyl or lead staining. × 15,000.

bodies, and at 2 hr the peroxidase reaction was still negative for most microbodies (Fig. 4). The microbody core appeared electron opaque against a lighter matrix and resembled closely cores in cytochemical controls incubated in the absence of hydrogen peroxide. In a few microbodies at 2 hr, the matrix seemed slightly more electron opaque than in other microbodies, suggesting a minimal positive reaction.

By 4 hr, peroxidase activity had clearly returned to many microbodies, although the intensity of staining was significantly lower than in untreated animals (Fig. 5). However, some microbodies still did not show obvious peroxidase activity.

At 6 and 8 hr, respectively, the peroxidase reaction of the microbody matrix was more intense than at earlier intervals, and by 8 hr few microbodies remained unstained (Fig. 6). Nevertheless, the intensity of staining remained somewhat less than that in normal hepatic cells.

During the early hours following the AT injection, ribosomal staining in close proximity to

microbodies was observed frequently as in untreated animals, a positive reaction also being present in the microbody matrix. However, as noted above, not all microbodies exhibited peroxidase activity, and it was noteworthy that ribosomal staining also was seen adjacent to some of these microbodies, particularly at intervals between 2 and 6 hr (Figs. 8 and 9).

Peroxidase activity was not detected within cisternae of the rough and smooth endoplasmic reticulum or the Golgi apparatus at any time, and peroxidase staining did not reveal continuities between these organelles and microbodies.

In contrast to its effects on the microbody matrix, the administration of AT did not affect the positive reaction for peroxidase in red blood cells, the azurophil granules of leukocytes, lysosomes of Kupffer cells, or peribiliary dense bodies.

DISCUSSION

The biochemical effects of 3-amino-1,2,4-triazole (AT) on liver catalase were investigated initially
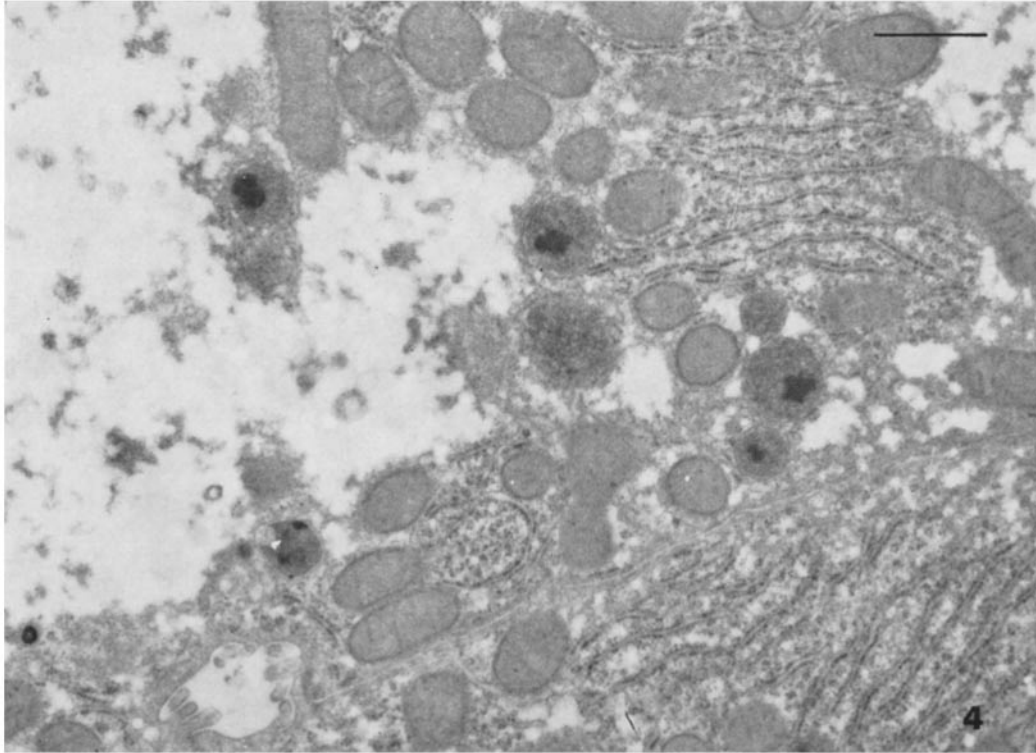
FIGURE 4 2 hr after AT injection, reacted for peroxidase activity. There is little or no reaction product in the microbodies, and the nucleoids are prominent. No uranyl or lead staining. × 15,000.

by Heim et al. (10, 11) who found that catalase activity was approximately 10% of normal at 3 hr after intraperitoneal injection. Price and co-workers (24) later extended these studies and reported that at 1 hr after the injection of AT only 2% of normal enzyme activity remained, attributable to red blood cell catalase; the activity then gradually increased, reaching normal levels approximately 5 days after injection. The present study demonstrates clearly that the inhibition of liver catalase activity after intraperitoneal injection of AT is directly paralleled by inhibition of microbody peroxidase-staining. Although it is still not entirely certain that the peroxidase activity observed in microbodies with the technique used is due to catalase, these results are in accord with the proposals of Essner (6), Fahimi (7), Hirai (13), and Venkatachalam and Fahimi (30) that catalase activity is indeed responsible for the observed reaction.

The mechanism of catalase inhibition by AT also was explored by Heim et al. (10, 11) who

found evidence for both reversible and irreversible inhibition. In vitro, catalase was inhibited only at high concentrations of AT, apparently owing to nonspecific denaturation of the catalase (8, 11), and the inhibition was partially reversible by dilution. On the other hand, in vivo inhibition occurred with much smaller quantities of AT, and the inhibition was not reversible. In this situation, AT forms an irreversible complex with catalase by binding to its protein moiety (19, 20, 23, 24).

In related studies, Price et al. (23, 24) demonstrated that the return of catalase activity following administration of AT in vivo was accompanied by a corresponding uptake of radioactive iron into the catalase. They concluded that the return of catalase activity was due to the synthesis of new catalase, and recent studies by de Duve and co-workers (22) add support to this conclusion.

The current evidence, therefore, indicates that the return of catalase activity after treatment with AT is due to the formation of new catalase rather than to reversal of inhibition. Since liver catalase
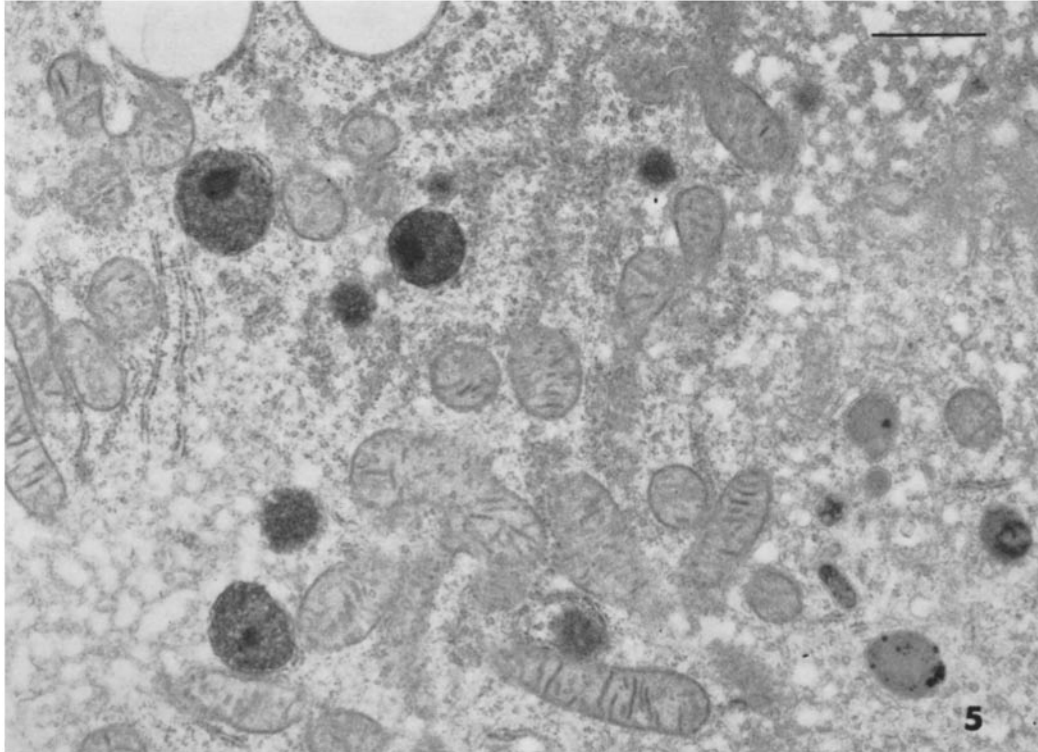
FIGURE 5 4 hr after AT injection, peroxidase reaction. The microbody matrix shows definite reaction product, but nucleoids remain obvious. The dense bodies at the lower right show a strong peroxidase reaction as in normal liver. No uranyl or lead staining. × 15,000.

is believed to be largely associated with microbodies (16), the newly synthesized catalase must either be sequestered only into newly formed microbodies or be capable of entry into preexisting microbodies. The present observations are more consistent with the latter interpretation. Throughout the duration of the experiment, there was no morphological evidence to suggest that the microbodies were either being formed or destroyed rapidly. At each sampling interval, most microbodies showed a similar intensity of peroxidase staining and there was an increase in the intensity of staining with increasing time after AT injection. Small microbodies with intense staining were not observed within the same hepatic cell that contained larger microbodies lacking peroxidase activity. This is contrary to what would be expected if catalase were being sequestered only into newly formed microbodies. Furthermore, some large microbodies with a well-formed core exhibited peroxidase activity as early as 2 hr after the injection of AT.

Thus, in the absence of evidence of either massive destruction of microbodies or rapid formation of new microbodies, the rapidity of return of enzyme activity following treatment with AT strongly suggests that newly synthesized catalase enters microbodies already present in the hepatic cell.

The manner in which the catalase might enter preexisting microbodies is an intriguing problem, and three possible mechanisms deserve consideration. First, after synthesis on the attached ribosomes (12), catalase may pass into the cisternae of the rough endoplasmic reticulum to be packaged by the Golgi apparatus, in a process similar to that demonstrated for other proteins (3). However, several features suggest that this may not be the preferred pathway. There is no evidence that catalase or the other microbody enzymes are secretory proteins, and, in fact, their secretion from the cell would be contrary to the "peroxisome concept" of de Duve (4). Hence, catalase does not necessarily have to be processed by the Golgi
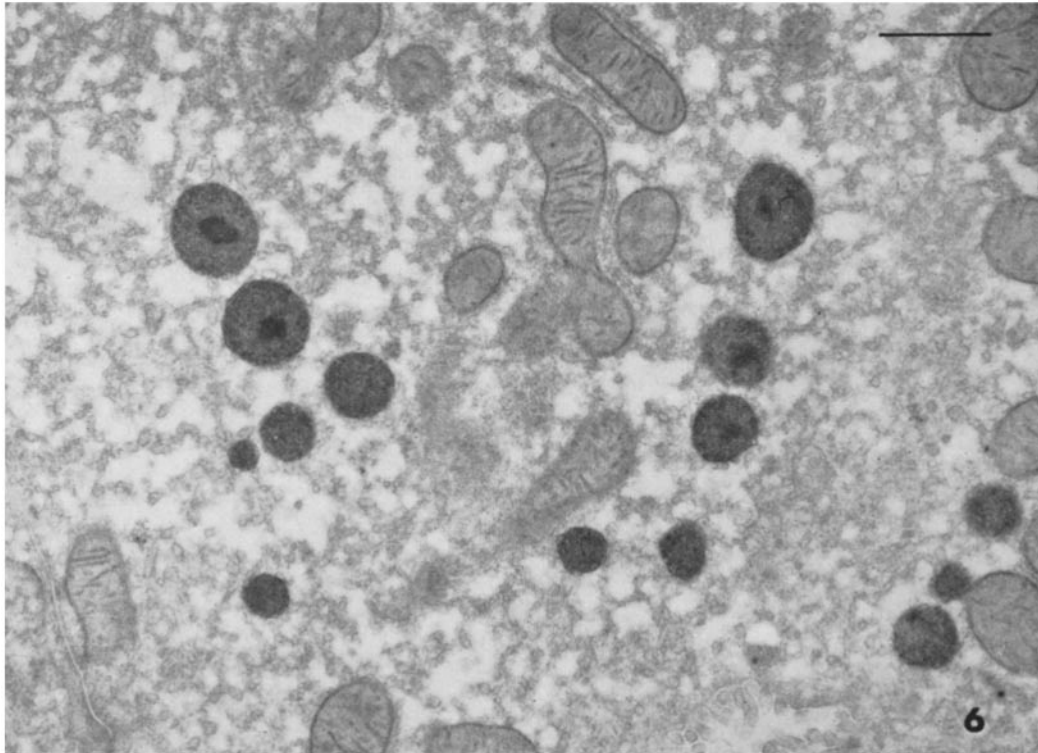
FIGURE 6   8 hr after AT injection, peroxidase reaction. The staining of the microbody matrix is more dense than at earlier times, but not so dense as in normal liver (see Fig. 1). No uranyl or lead staining. × 15,000.

apparatus as is a conventional secretory protein. Furthermore, the morphological and cytochemical results from this and other studies provide little indication that the Golgi apparatus is actually involved in microbody formation. Finally, the evidence considered earlier strongly suggests that catalase formed after AT inhibition is capable of entering preexisting microbodies and is not restricted to newly formed microbodies.

The second possibility is that direct passage of catalase may occur from within cisternae of rough or smooth endoplasmic reticulum into microbodies. Such a mechanism would necessitate at least transitory membrane continuity between these organelles and microbodies. Despite the assertions of previous workers (5, 21, 29), careful examination in this and previous studies in our laboratories (15) have failed to demonstrate such continuities convincingly and have not confirmed this concept.

Thirdly, catalase might be transferred directly into the microbodies without segregation into the

cisternae of the rough endoplasmic reticulum at any stage (15). The rapid return of catalase activity to the microbody after AT administration without evidence for rapid proliferation or destruction of microbodies, the lack of evidence for membrane continuity between microbodies and endoplasmic reticulum, and the failure to detect peroxidase staining within either the endoplasmic reticulum or the Golgi apparatus are more consistent with this interpretation. The mechanism by which the direct transfer of catalase across the microbody membrane might take place still remains uncertain, but it could involve lower molecular weight enzymatically active subunits of the enzyme (15) or migration of the heme and protein moieties separately.

Ribosomal staining adjacent to microbodies was noted in an earlier study (15), and it was suggested that this might reflect the site of synthesis of catalase prior to transfer into microbodies. In the present study, ribosomal staining was observed associated with microbodies whose matrix showed
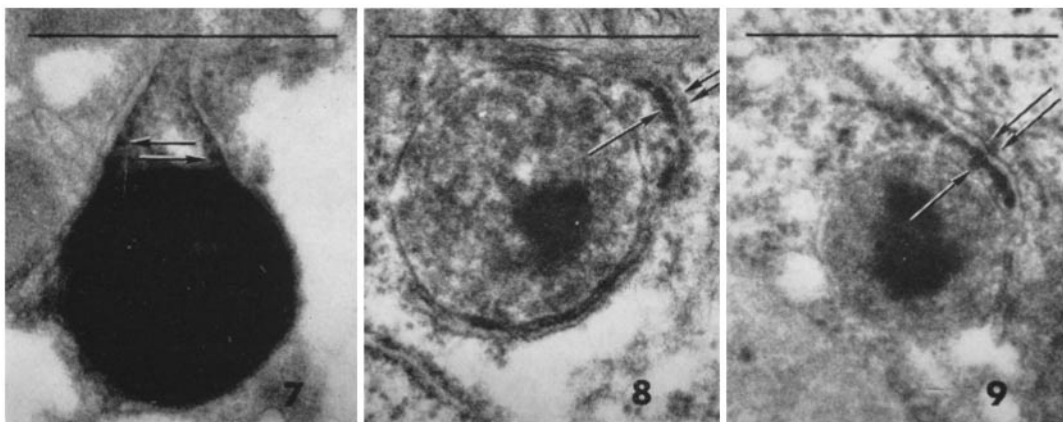
FIGURE 7 Microbody in normal hepatic cell, peroxidase reaction, showing strong activity of the microbody matrix. Ribosomal staining is prominent (arrows). No reaction product is present in the cisterna of the rough endoplasmic reticulum. No uranyl or lead staining. × 41,000.

FIGURES 8 and 9 Microbodies in treated hepatic cells, peroxidase reaction, 4 hr after AT (Fig. 8) and 6 hr after AT (Fig. 9). Little or no reaction product is present within the microbody matrix. The electron opacity of the prominent nucleoid is comparable to that seen in controls. Ribosomes on adjacent rough endoplasmic reticulum show a positive reaction (single arrows), whereas on the opposite side of the same cisterna ribosomes show no reaction (double arrows). In Fig. 8, staining is associated also with adjoining surfaces of the endoplasmic reticulum and limiting membrane of the microbody. No uranyl or lead staining. × 41,000.

little or no peroxidase staining. It seems unlikely that the ribosomal staining in the absence of microbody staining could be diffusion artifact since this phenomenon is usually associated with heavy deposits of reaction product. With the knowledge that catalase synthesis is proceeding during the early hours after AT injection, it seems reasonable to relate ribosomal staining with the synthesis or the site of activation of catalase. It is of interest that AT fails to inhibit this ribosomal staining. This implies that all the injected AT within a few hours is made unavailable for reaction with newly formed catalase.

The fate of the irreversible complex of AT and catalase, presumably formed in the microbody, cannot be ascertained at present. There was no obvious difference in morphological appearance of microbodies in AT-treated compared with normal hepatic cells. From biochemical studies, Price et al. (24) deduced that the inactive catalase progressively disappears from the livers of animals treated with AT, but whether the complex is

destroyed within the microbody matrix or migrates outside the microbody is not known.

The present study provides further information on the intriguing possibility that at least one component of the microbody, catalase, may be capable of sequestration into the organelle without involving transit through the cisternae of the endoplasmic reticulum or the Golgi apparatus. Poole et al. (22) suggested that catalase turnover may represent the turnover of entire microbodies, but it seems hardly conceivable that the entire population of liver microbodies could be replaced in the first few hours after AT injection. The present data do not support this suggestion.

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