EFFECT OF BUFFER STORAGE ON FINE STRUCTURE AND CATALASE CYTOCHEMISTRY OF PEROXISOMES

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Peroxisomes are morphologically characterized by a single limiting membrane and a finely granular matrix, and some of them contain crystalline nucleoids (1, 2). Application of the alkaline 3,3'-diaminobenzidine (DAB) method, which visualizes the peroxidatic activity of catalase (3, 4) in normal liver, has revealed electron-dense reaction product in the matrix of peroxisomes, but none in the cytoplasm (5, 6). Legg and Wood and associates, on the other hand, have described evidence of staining of ribosomes and the endoplasmic reticulum membranes adjacent to peroxisomes in liver of normal and treated rats in conditions associated with increased *de novo* synthesis of catalase (7–10). Although these authors raised the possibility that diffusion artifacts might cause the ribosomal staining, they considered it unlikely since "under all conditions used, microbodies with sharp localization of reaction product were present in the same sections that showed microbodies with adjacent ribosomal staining" (7). Some of these observations were also corroborated in preliminary studies, from ours as well as various other laboratories (11–15). Nevertheless, Novikoff et al., in discussing the various causes of diffusion artifacts in DAB cytochemistry (16), concluded that the ribosomal staining adjacent to peroxisomes is due to diffusion of oxidized DAB, which is generated within the peroxisomes and subsequently diffuses out and is adsorbed on ribosomes (16, 17).

Recently, we studied the various causes of diffusion artifacts in the cytochemistry of catalase and demonstrated that indeed the ribosomal staining is due to *diffusion of catalase* rather than oxidized DAB (18). Such diffusion occurs in the course of rinsing or storage of tissue sections in buffer after aldehyde fixation and before incubation in DAB medium (18). The present communication deals with two questions: (*a*) what is the effect of buffer storage upon the fine structure of peroxisomes; and (*b*) does exposure to buffer affect all peroxisomes uniformly, or is there a heterogeneous response, with some peroxisomes exhibiting diffusion and other adjacent particles within the same cell exhibiting none?

MATERIALS AND METHODS

Animals

Male adult albino rats of the Charles River strain (CDR) weighing 250–350 g and fed a normal diet and water ad libitum were used. The animals were fasted for 16 h before sacrifice in order to decrease the content of hepatic glycogen.

Fixation

All livers were fixed by perfusion through the portal vein as described previously (5). The fixative contained 2.5% distilled glutaraldehyde (Ladd Industries, Burlington, Vt.) in 0.1 M cacodylate buffer pH 7.2, and 0.01% calcium chloride. The perfusion was carried out at room temperature for at least 10 min at a flow rate of 15–20 ml of fixative per minute. The quality of fixation was assessed grossly (19), as well as by light and electron microscopy, and only uniformly well-fixed livers were used.

Storage of Tissues in Buffer

Immediately after fixation, small blocks of liver measuring 10 × 1 × 2 mm were cut into 30-μm sections with a TC-2 Smith-Farquhar tissue chopper (20) (Ivan Sorvall, Inc., Norwalk, Conn.). From each animal, some sections were processed *immediately* for fine structural

and cytochemical studies, whereas other sections were first stored at 4°C in various buffers and aqueous media for different time intervals ranging from 18 h to 1 wk and were processed subsequently. The following media were used for storage of sections: 0.1 M cacodylate with and without 5% sucrose; 0.15 M cacodylate buffer; 0.1 M Tris-HCl buffer; and 0.1 M phosphate buffer, all at pH 7.2. In addition, some sections were stored in distilled water and in isotonic (0.85%) saline.

Postfixation

Three methods were used: (*a*) 2% aqueous osmium tetroxide for 90 min at room temperature; (*b*) the same, followed by 1 h in 1% uranyl acetate in 0.2 M hydrogen-maleate buffer at pH 5.2 (21); (*c*) 3% aqueous potassium permanganate for 30 min at room temperature. All sections were then rapidly dehydrated in cold ethanol and embedded in Epon (22).

Incubation for Catalase

The incubation medium contained 0.1% DAB dissolved in 0.1 M Tris buffer with the final pH adjusted to 8.5, and 0.02% H_2O_2 (5). Sections were preincubated for 30 min without H_2O_2, and for 60 min in complete medium at 37°C. In control experiments, parallel sections were incubated in the same medium which, in addition, contained 2×10^{-2} M of 3-amino-1,2,4-triazole. After incubation, sections were postfixed for 90 min in 2% osmium tetroxide, using for some sections the reduced osmium procedure of Karnovsky (23), followed by rapid dehydration and embedding in Epon (22).

Microscopy

1-μm thick sections were examined by light microscopy and from selected areas; ultrathin sections were cut with a diamond knife on an LKB Ultratome III microtome and were examined either unstained or lightly counterstained with lead citrate (24) in a Philips EM 200 electron microscope. In addition, ribbons of ultrathin serial sections were prepared and placed on grids with a single central slot previously coated with 1% Parlodion and a thin layer of carbon, and were stained with lead citrate. Electron micrographs were obtained from the same regions of 10–12 consecutive sections.

RESULTS

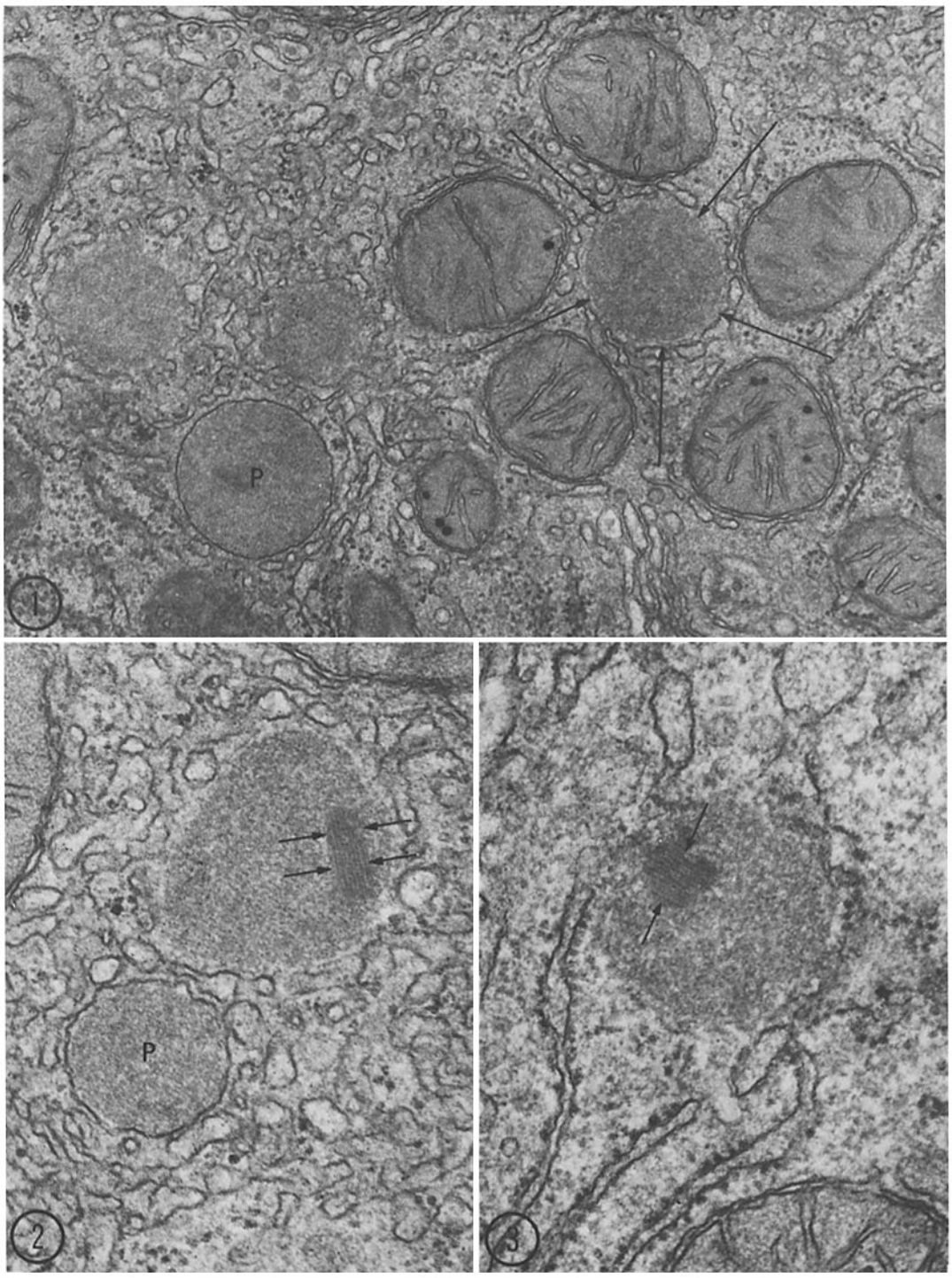
Fine Structural Observations

In material which was postosmicated immediately after the glutaraldehyde fixation, *all* peroxisomes had the conventional appearance with a single limiting membrane and a finely granular matrix, and some of them contained crystalline nucleoids (1, 2). In contrast, in sections which were stored in buffer for 18 h or longer, in addition to normal peroxisomes with distinct membranes side by side within the same cell, there were particles which had the same shape and matrical density as peroxisomes but which lacked a distinct limiting membrane (arrows, Fig. 1). Some of these peroxisomes with poorly demonstrable membranes contained crystalline nucleoids (Figs. 2, 3). Treatment of sections with uranyl acetate en bloc improved the general contrast of most membranes, but there were still many peroxisomes with poorly distinguishable or indistinguishable limiting membranes next to normal-appearing peroxisomes with distinct membranes.

In material which was postfixed with potassium permanganate immediately after glutaraldehyde fixation, the limiting membranes of all peroxisomes, as well as other cytoplasmic membranes, appeared well preserved and distinctly electron dense. However, after the storage of tissue in buffer, in addition to normal peroxisomes, there were again many particles with the matrical density of peroxisomes which lacked a distinct limiting membrane (Figs. 4, 5). By careful examination, some of these particles, however, appeared to be partially surrounded by short discontinuous segments of membrane (particle A in Fig. 4 and

FIGURE 1 Section of rat liver fixed by glutaraldehyde perfusion, chopped, and stored for 72 h in 0.1 M cacodylate buffer pH 7.2, followed by postosmication and processing for electron microscopy. In addition to normal peroxisomes with distinct membranes (*P*), there are particles which have the same shape and matrical density as peroxisomes but which lack a clearly distinguishable limiting membrane (arrows). Counterstained with lead citrate. × 34,500.

FIGURES 2, 3 These figures are from rat liver fixed by glutaraldehyde perfusion, chopped, and stored for 72 h in 0.15 M cacodylate buffer, followed by postosmication and treatment with uranyl acetate en bloc (21). Note in Fig. 2 a peroxisome with a distinct membrane (*P*) next to a particle containing a crystalline nucleoid (arrows) but lacking a distinguishable membrane. × 63,000. Similarly, in Fig. 3 there is a particle with a nucleoid (arrows) and the matrical density of peroxisomes which lacks a distinct membrane. × 69,000.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1
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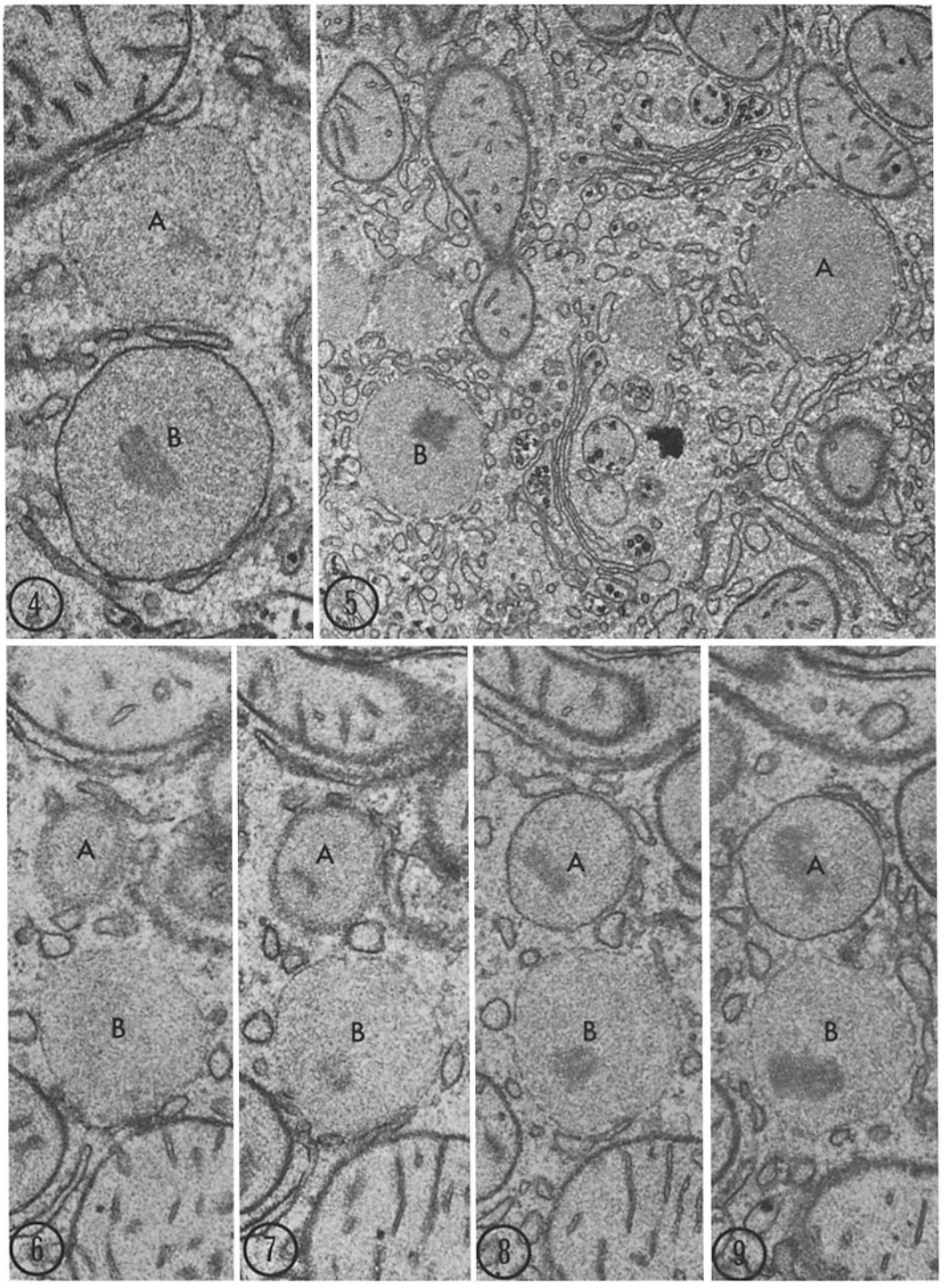
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particle B in Fig. 5). To rule out the possibility that the appearance of peroxisomes without membranes could be the result of tangential sectioning of regular peroxisomes, this material was examined in serial sections. Figs. 6–9 are four selected views from 10 serial sections which demonstrate side by side a peroxisome with a membrane (particle A), and a particle with a poorly demonstrable and discontinuous membrane (particle B). Particle A has been cut tangentially in Fig. 6, and therefore its limiting membrane is not clearly visible, but in subsequent sections this membrane becomes distinctly demonstrable (Figs. 8, 9). In contrast, the limiting membrane of the larger particle B is difficult to distinguish and appears discontinuous at all four levels (Figs. 6–9).

It should be emphasized that in osmium tetroxide- and permanganate-fixed sections, the membranes of the Golgi apparatus and of the rough and smooth endoplasmic reticulum located in the vicinity of peroxisomes without membranes were well preserved and did not seem to be affected by the storage of tissues in buffer (Figs. 1–3 and 5).

Cytochemical Observations

In material incubated immediately after fixation, the reaction product of oxidation of DAB was confined to the matrix in all peroxisomes and there was no evidence of diffusion beyond the limiting membrane. In contrast, when the incubation was carried out after the storage of sections in buffer, there was evidence of diffusion in the vicinity of some peroxisomes, whereas adjacent microbodies exhibited no diffusion (Fig. 10). At higher magnification, the reaction product around such diffusing or "leaky" peroxisomes was localized on membranes of the endoplasmic reticulum as well as on free and membrane-bound ribosomes (Fig. 11). Such evidence of diffusion after the storage of sections in buffer was also seen in material postfixed with the reduced osmium method of Karnovsky (23). The intensity of staining for catalase diminished with increasing diffusion, and most of the particles with severe diffusion appeared to lack a distinct limiting membrane (Figs. 11, 12). The staining of both types of peroxisomes with and without diffusion was inhibited by 2×10^{-2} M of aminotriazole.

Effect of the Composition of the Storage Medium and Other Variables on Peroxisomes

Most of the observations reported here were made with tissues stored in 0.1 or 0.15 M cacodylate buffer. Since the diffusion of catalase was easier to observe than the presence or absence of membranes around peroxisomes, the effect of various buffers and other variables was assessed only in cytochemical preparations. Thus, catalase diffusion was observed in glutaraldehyde-fixed material stored in phosphate and Tris buffers as well as in sections stored in isotonic saline and distilled water. The addition of sucrose to the 0.1 M cacodylate buffer decreased slightly the severity of the diffusion but did not completely prevent it. The severity of diffusion and the number of peroxisomes exhibiting it increased with prolongation of the time of storage of tissue in buffer. Furthermore, there was more diffusion of catalase in cells on the surface of tissue blocks which were directly exposed to the rinsing medium than in cells deeper within the block. This variation in different cells and different parts of the block

FIGURES 4–9 These are all from material postfixed with 3% aqueous potassium permanganate.

FIGURES 4, 5 These figures illustrate the appearance of peroxisomes in material which was stored for 72 h in 0.15 M cacodylate buffer and subsequently postfixed with permanganate. In addition to peroxisomes with distinct membranes (particle *B* in Fig. 4), there are particles with the same size and shape but with a discontinuous and poorly preserved limiting membrane (particle *A* in Fig. 4, and particles *A* and *B* in Fig. 5). By careful examination, short discontinuous segments of a somewhat thinner membrane can be seen around some of these particles (particle *A* in Fig. 4, and particle *B* in Fig. 5). Fig. 4, × 38,500. Fig. 5, × 26,600.

FIGURES 6–9 These are selected views from 10 serial sections which illustrate side by side a peroxisome with a membrane (particle *A*) and a particle with the same shape and matrical density but with a discontinuous and poorly visible membrane (particle *B*). Particle *A* has been cut tangentially in Fig. 6 and therefore its limiting membrane is not clearly visible, but in deeper sections it becomes distinctly demonstrable (Figs. 8, 9). In contrast, particle *B* shows at all four levels no (or only short) segments of discontinuous membrane. Note the presence of nucleoids in both particles, which confirms their identity as peroxisomes. Figs. 6–9, × 29,600.

interfered with attempts to quantitate exactly the proportion of peroxisomes showing diffusion. However, random counts of 150–250 particles from several animals revealed that after 18 h of storage of chopped sections in buffer, approximately 40–60% of peroxisomes exhibited diffusion. This number increased with increasing time of diffusion, but even in cells with severe diffusion, a few peroxisomes (approximately 5–10%) remained refractory and did not exhibit any evidence of diffusion.

DISCUSSION

The observations reported here indicate that storage of glutaraldehyde-fixed sections of rat liver in various buffers and aqueous media results in progressive deterioration of peroxisome structure, as evidenced by the disappearance of morphologically recognizable membrane and by the diffusion of cytochemically detectable catalase from the matrix of peroxisomes into the adjacent cytoplasm. These observations concur basically with biochemical data which indicate that the microbody membrane is very fragile and that freshly isolated peroxisomes release readily their catalase content after exposure to various physical and chemical treatments such as prolonged washing (25), excessive homogenization (26), osmotic shock (27), alkaline pH (28), and detergents (1, 27, 29, 30). In particular, the progressive disruption of the microbody membrane in our studies seems to correlate well with the observations of Baudhuin (27) that storage of (unfixed) peroxisome fractions leads to progressive elution of catalase from the particles. On the other hand, Baudhuin (27) and Leighton et al. (28) have also shown that incubation of freshly isolated peroxisomes at 37°C and their treatment with alkaline buffer at pH 9.0 cause severe elution of catalase from the particles. These are conditions which are usually used for cytochemical demonstration of peroxidatic activity of catalase (5, 6). Nevertheless, recent findings from this laboratory and others demonstrate that incubation at even higher pH values of 9.7 (31) and 10.5 (32), as long as it is carried out shortly after glutaraldehyde fixation, does not cause any diffusion of catalase from peroxisomes. Further, the diffusion does not seem to be related to the type of postosmication (33) since it was seen both in material treated with regular osmium tetroxide and in material treated with reduced osmium tetroxide (23).

Our results show that the prolonged storage of sections in buffer *before incubation* is responsible for the diffusion of catalase from peroxisomes and thus accounts for the staining of ribosomes and cytoplasmic membranes adjacent to peroxisomes (7–15). Although catalase antigen has been demonstrated on free and membrane-bound ribosomes of rat liver by immunochemical methods (34, 35), recent studies by Lazarow and de Duve indicate that this nascent catalase lacks enzymatic activity and that the enzyme gains its full activity within the peroxisomes (36, 37).

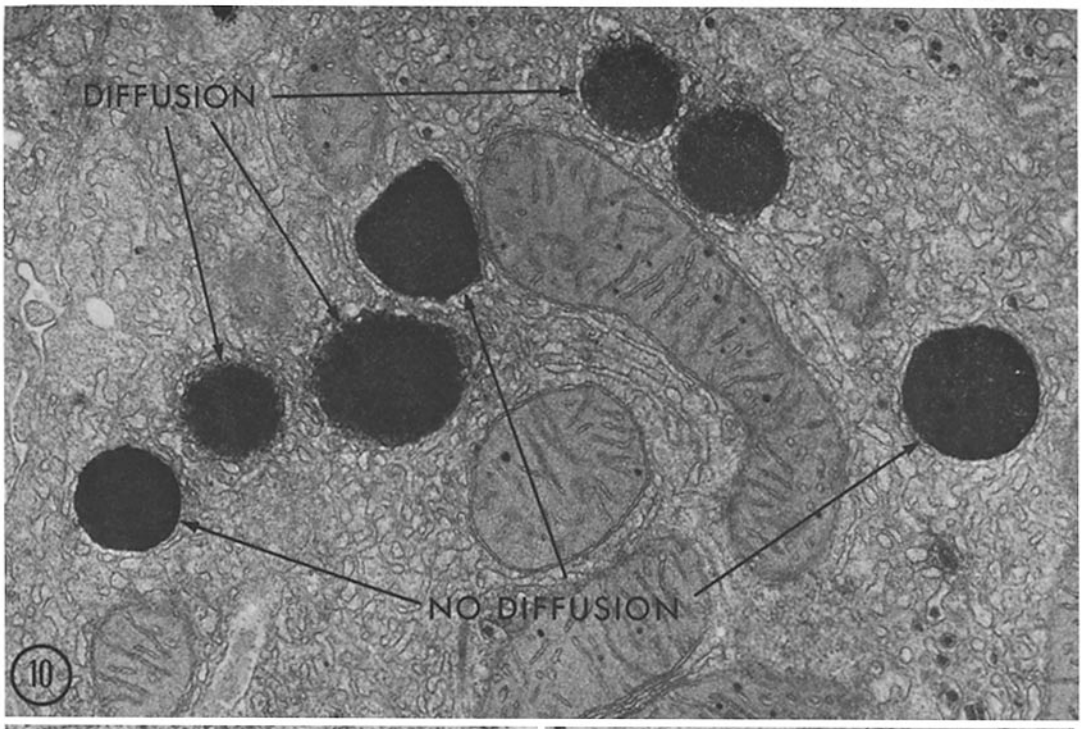
Although glutaraldehyde is a well-known cross-linking agent for proteins, the reactions between glutaraldehyde and lipids have not been extensively explored (38). Levy et al. (39) reported that glutaraldehyde apparently did not react chemically with lipids of the brain tissue and did not change their solubility characteristics during chloroform-methanol extraction. Roozemond (40) noted that

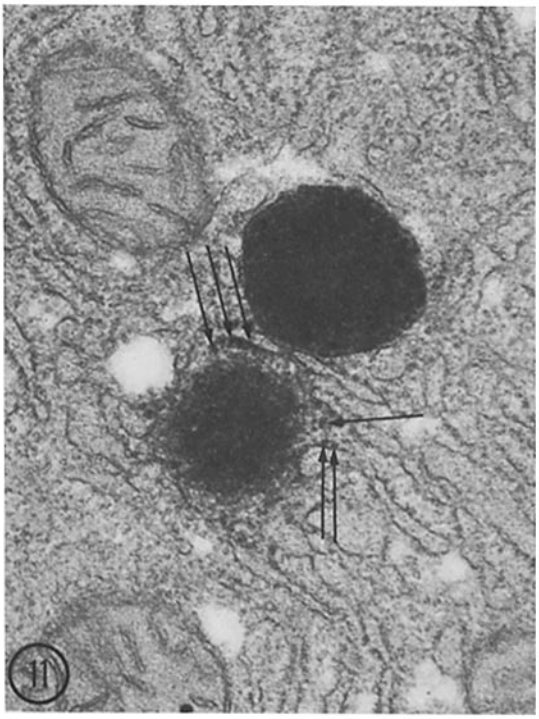
FIGURES 10–12 These figures are from rat liver fixed by perfusion with glutaraldehyde, chopped, and stored for various time intervals in buffer, and subsequently incubated in DAB medium (5).

FIGURE 10 In this electron micrograph two distinct types of peroxisomes are seen: one group exhibiting diffusion of catalase into the adjacent cytoplasm (*Diffusion*), and the other group showing no evidence of diffusion (*No Diffusion*). × 25,900.

FIGURE 11 In this figure a peroxisome with diffusion is shown adjacent to a peroxisome without diffusion. Note the prominent staining of ribosomes adjacent to the particle with diffusion (arrows), and note the lack of a distinct membrane around this particle. × 45,300.

FIGURE 12 This figure is from the material stored for 1 wk in buffer before incubation and shows a peroxisome with severe diffusion (arrows) next to a peroxisome with no diffusion (*P*). Note the decreased intensity of reaction and the apparent absence of a distinct membrane around the particle with diffusion. × 35,300.


DIFFUSION
NO DIFFUSION
10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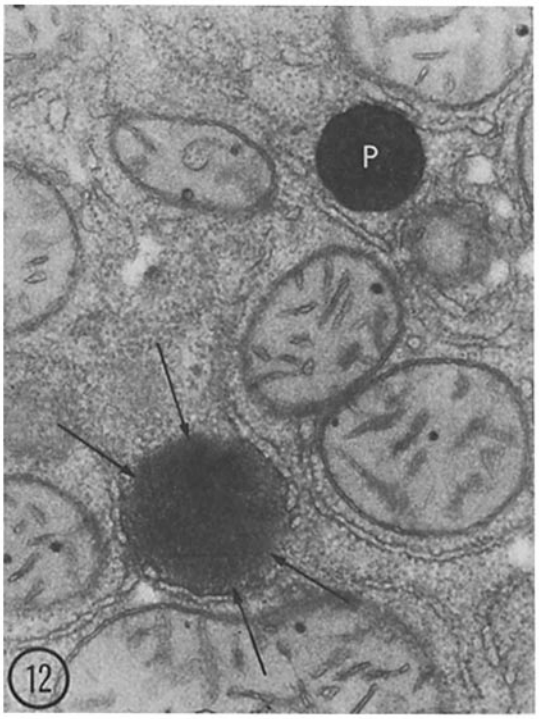
P
12

glutaraldehyde reacted mostly with those phospholipids which contained free amino groups, such as phosphatidylserine and phosphatidylethanolamine, but did not fix and prevent the extraction of phosphatidylcholine. Donaldson, Tolbert, and Schnarrenberger, however, have recently found that phosphatidylcholine makes up 55% of the total lipid of isolated microbody membranes (41). This may explain the lability of the microbody membranes in glutaraldehyde-fixed tissues.

In the present study it was consistently observed that peroxisomes with and without morphologically distinguishable membranes and cytochemically demonstrable diffusion of catalase were located next to each other within the same cell; this cytochemical observation confirms the findings of Legg and Wood (7). Although the effect of buffer storage on microbody membrane and the effect of buffer storage on catalase diffusion may be two completely unrelated phenomena, the absence of a clearly visible membrane around the microbodies which exhibited severe diffusion (Figs. 11, 12) would suggest that probably the catalase diffusion occurs secondarily to the damage to the microbody membrane. Finally, it should be noted that, although the differences in the susceptibility of different peroxisomes to buffer storage in our preparations may be purely randomly occurring phenomena, they could also reflect differences in the fragility of peroxisomes in vivo. According to this hypothesis, one could speculate that particles with more vulnerable membranes would be the source of the so-called "extraparticulate" or cytoplasmic catalase, and that the more stable peroxisomes would be the source of the "particulate" or peroxisomal catalase (42–44). The validity of this hypothesis can easily be assessed since the ratio of particulate catalase to extraparticulate catalase is supposed to be fairly constant in different animal species and among the different tissues of the same animal (44).

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