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MEMBRANE STRUCTURE AND FUNCTION

Edited by

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Preface

This volume addresses the subject of organelles. Its aim is to give the reader a view of recent research progress in the field of peroxisomes and mitochondria. That signal progress has been made cannot be doubted. However, knowledge of organelle organization and function as well as of organelle interaction has not advanced far enough to provide us with a critical understanding of how the cell maintains order and how it does so *in vivo*. It is an article of faith that the problem is not an insoluble one. The problem remains the most fundamental with which cell biology has yet to deal.

My most grateful thanks are due to the authors who have made these volumes in the series possible, and to the staff of Wiley for their skill and cooperation. Special thanks are also due to Dr. Barbara Littlewood for preparing the indexes.

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Madison, Wisconsin
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CHAPTER ONE

The Peroxisomal Membrane

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1 INTRODUCTION

This review describes the procedures that have been devised for the isolation of the peroxisomal* membrane and then summarizes the knowledge that has been thereby gained about the composition and properties of this endomembrane. The rest of the article deals with morphology and biogenesis. Attention is focused first on the peroxisomal membrane in rat liver and then on peroxisomal and glyoxysomal* membranes of plants. Finally, the plant and animal data are compared.

2 MEMBRANE OF RAT LIVER PEROXISOMES

2.1 Isolation Procedures

Two principal procedures have been described for the isolation of the peroxisomal membrane from rat liver. Both begin with the purification of peroxisomes by sequential differential and equilibrium density centrifugation, generally in sucrose media as desctibed by Leighton et al. (9) or with slight modifications. Isolated peroxisomes are illustrated in Figure 1*a*. Most peroxisomes retain their contents, but a few have lost most of

* Several terms are used to describe this organelle. A *peroxisome* is defined biochemically as an organelle containing at least one hydrogen peroxide-producing oxidase and catalase (1). Peroxisomes have a wide variety of functions in the various cell types in which they are found; see refs. 2–4 for recent reviews. A *glyoxysome* is a specialized type of peroxisome containing the enzymes of the glyoxalate cycle (5). *Microbody* is a morphological term, originally used to describe structures approximately 0.5 μm in diameter with a uniform matrix, bounded by one membrane, and sometimes containing a core (6, 7). Structures that fit this morphological definition include peroxisomes and glyoxysomes as well as some biochemically unrelated particles (8).

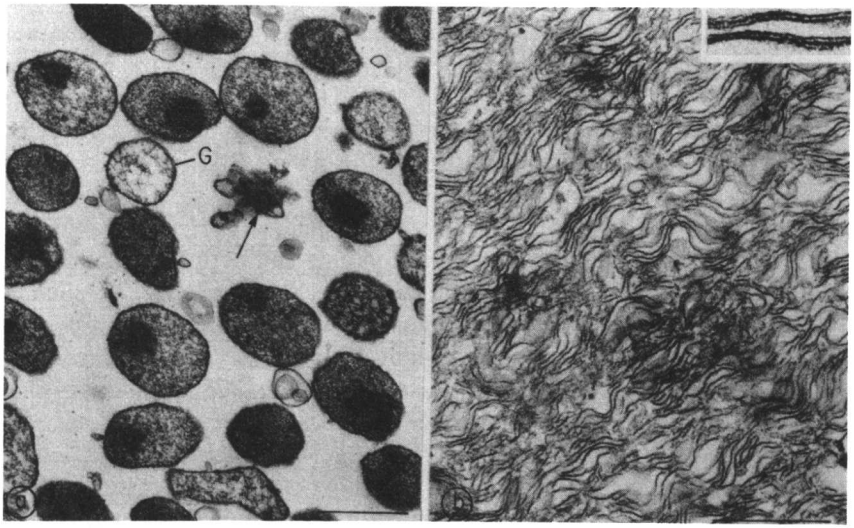

Figure 1 Electron micrographs of purified rat liver peroxisomes (*a*) and peroxisomal membranes prepared by sodium carbonate treatment (*b*). G = ghost of peroxisome. Arrow indicates a free peroxisomal core. Bar, 0.5 μm. Inset: bar, 50 nm. From ref. 13.

their matrix proteins and become "ghosts." One such ghost is identified with a G in Figure 1*a*.

The purity of the peroxisomal fraction is the main factor in determining the purity of the subsequent membrane preparations; evaluation of purity is discussed in Section 2.2.

Donaldson et al. (10) and Huttinger et al. (11) treated isolated peroxisomes with 10 m*M* pyrophosphate buffer, pH 9, essentially as described by Leighton et al. (12) and then centrifuged the treated peroxisomes into a discontinuous sucrose gradient. This procedure shocks the peroxisomes osmotically, and in addition the alkalinity of the pyrophosphate medium itself contributes to the release of matrix proteins (12). Both groups reported that recentrifugation yielded a soluble matrix fraction at the top of the gradient, a urate oxidase-containing core fraction near the bottom—and in between a membrane fraction. Huttinger et al. (11) found by electron microscopy that the membranes consisted of closed vesicles with diameters mainly between 100 and 300 nm. Some vesicles contained cores, and many had residual fibrillar contents; this membrane fraction consists of peroxisomal ghosts very much like the one labeled G in Figure 1*a*.

Fujiki et al. (13) treated isolated peroxisomes with 0.1 *M* Na_2CO_3 (14) and found that centrifugation at 50,000 rpm yielded a pellet of membranes that consisted of flat, unsealed sheets (Figure 1*b*) in which the trilaminar nature of the unit membrane was still visible (inset). Control experiments on microsomal fractions showed that the carbonate procedure converted microsomal vesicles to unsealed sheets (14), which retained their integral proteins (the ribophorins and cytochromes b_5 and P_{450}), active integral enzymes (NADPH–cytochrome *c* reductase and NADH-ferricyanide reductase), and phospholipid. Cisternal contents and peripheral membrane proteins were released. Comparison with other methods (15–17) indicates that the carbonate procedure distinguishes between integral and peripheral membrane proteins. Peroxisomal membranes prepared by carbonate treatment contain 12% of the peroxisomal protein, 77% of the phospholipid, and only one of the major polypeptides visible among total peroxisomal proteins in SDS–polyacrylamide gel electrophoresis in gradient slab gels (molecular mass = 21,700 daltons) (13).

In a variation on the first procedure for preparing peroxisomal membranes, Appelkvist et al. (18) isolated peroxisomes in a Percoll gradient from postnuclear supernatants that had been fixed with glutaraldehyde. The peroxisomal fraction was treated with 50 m*M* KCl/50 m*M* Tris-HCl, pH 7.5/0.05% deoxycholate, and centrifuged for $10^7 g$-min. Electron microscopy of the pellet revealed peroxisomal ghosts containing cores and fibrillar material; these resemble the membrane fraction of Huttinger et al. (11).

Fowler et al. (19) and Remacle (20) analyzed peroxisomes (isolated by sucrose gradient centrifugation) for cytochrome b_5 content by electron microscopic immunocytochemistry (see below). Although there was no intention of disrupting the peroxisomes, the immunochemical manipulations involved dilutions and recentrifugations; the result was the formation of peroxisomal ghosts very much like those in the membrane fractions described above (11, 18).

The osmotic shock/pyrophosphate procedure and the carbonate method are complementary. The former produces closed ghosts which retain peripheral membrane proteins as well as some of their contents. The latter yields open-membrane sheets containing only integral membrane proteins. The two procedures together should yield considerable useful information about the properties and organization of the peroxisomal membrane. However, since the peroxisomal ghost preparations contain some internal fibrillar material and cores, it does not necessarily follow that all enzymes found in ghosts are membrane-associated. Certain peroxisomal proteins might aggregate or otherwise be difficult to extract

from peroxisomes; these could account for some enzymatic activities of the ghosts.

2.2 Purity

The purity of peroxisomal membranes depends on the purity of the peroxisomes from which they are prepared. Accurate evaluation of purity is essential to meaningful comparisons with other types of membranes. Marker enzyme analyses have proven to be a sensitive and quantitative means of assessing purity. Ninety-five percent pure rat liver peroxisomes have a catalase-specific activity that is 36 times the homogenate specific activity (9). With care to avoid damage due to hydrostatic pressure, mitochondrial contamination of peroxisomes should be minimal because of the difference in their isopycnic densities. Cytochrome oxidase has generally been used as the marker enzyme for mitochondria. Contamination by microsomes is a much more difficult problem because rough microsomes are as dense as peroxisomes and therefore must be removed in the differential centrifugation steps preceding the sucrose gradient centrifugation. Glucose-6-phosphatase (10, 11, 18, 20) or esterase (13) are useful markers because they are completely absent from peroxisomes. Contamination (C) of peroxisomes by microsomes can be calculated in two straightforward ways:

1 $$C = \frac{\text{Glucose-6-phosphatase specific activity in peroxisomes}}{\text{Glucose-6-phosphatase specific activity in microsomes}} \times 100$$

assuming that the microsomes are pure.

2 $$C = \frac{\text{Glucose-6-phosphatase specific activity in peroxisomes}}{\text{Glucose-6-phosphatase specific activity in homogenate}} \times 20$$

since microsomes represent 20% of homogenate protein (9, 21, 22).

These calculations assume that there has been no differential loss of enzymatic activity during the fractionation, which requires a complete balance sheet and good recoveries to verify.

The contamination problem is aggravated when peroxisomal membranes are prepared because membranes represent a much smaller fraction of total organelle protein for the case of peroxisomes than for mitochondria or especially for microsomes (Table 1).

2.3 Composition of Rat Liver Peroxisomal Membranes

2.3.1 Enzymes

A number of investigators have reported the presence in peroxisomes of several electron transport enzymes similar to those in the endoplasmic

Table 1 Amounts of Organelle Membranes in Rat Liver[a]

	Organelle Protein[b] (mg/g liver)	Fraction of Organelle Protein in Membrane(s)[c]	Organelle Membrane Protein (mg/g liver)
Endoplasmic reticulum	52	0.53	28
Mitochondria	52	0.21	11
Peroxisomes	6.5	0.12	0.8

[a] From ref. 13

[b] Assuming 260 mg protein/g fasted liver, of which ER, mitochondria, and peroxisomes contribute 20, 20, and 2.5%, respectively.

[c] Membranes prepared by carbonate procedure (14).

reticulum (ER) (Table 2). Donaldson et al. (10) were the first to describe the presence of NADH–cytochrome *c* reductase and NADH–cytochrome b_5 reductase in peroxisomes. However, the 35% contamination by ER (estimated from their glucose-6-phosphatase data) was more than sufficient to account for the reductase activities.

Fowler et al. (19) constructed a hybrid anti-cytochrome b_5–anti-ferritin antibody, bound it to ferritin, and probed the location of cytochrome b_5 in various purified subcellular organelles by means of electron microscopic immunocytochemistry. Cytochrome b_5 labeling was abundant in ER and outer mitochrondrial membranes and sparse but yet greater than controls on peroxisomes, leading the authors to conclude that "peroxisomes may possess some cytochrome b_5 on their membranes." One of the advantages of this experimental approach was that the microsomal contaminants in the purified peroxisome fraction could be distinguished morphologically. Although the microsomal contaminants were much more highly labeled with ferritin, this did not interfere with the analysis of the labeling of the peroxisomes themselves. The relative specific activity of NADH–cytochrome *c* reductase was equal to that of glucose-6-phosphatase in these peroxisomes, indicating that the presence of reductase could be accounted for by microsomal contamination.

This approach was pursued by Remacle (20) who confirmed the presence of small amounts of cytochrome b_5 in peroxisomal membranes by EM immunocytochemistry with anti-cyt b_5 F(*ab*) fragments chemically coupled to ferritin. Moreover, he showed that purified holo-cytochrome b_5 could be incorporated into the peroxisomal membrane in vitro (up to 2 nmol/mg protein from an endogenous level of 0.01) where it was 87%

Table 2 Biochemical Characterizations of Peroxisomal Membrane Preparations from Rat Liver

	Reference Number	NADH–Cyt *c* Reductase (nmol/min / mg protein)	Cyt b_5 (nmol / mg protein)	NADH–Cyt b_5 Reductase (nmol/min / mg protein)	Glucose-6-Phosphatase (nmol/min / mg protein)	Esterase (nmol/min / mg protein)	Contamination by Microsomes (%)
1. Donaldson et al.	10						
Peroxisomes		310		5.2	39		35
Microsomes		1470		21.8	112		
Perox/Mic		0.21		0.24	0.35		
2. Fowler et al.	19						
Peroxisomes			+				11
Microsomes			+ + + +		370[a]		
Perox/Homogenate		0.57			0.57		
3. Remacle	20						
Peroxisomes			0.01	+			1
Microsomes			0.46		370[a]		
Perox/Mic		0.23	0.02				
Perox/Homogenate		0.69			0.05		
4. Huttinger et al[b]	11						
Peroxisomes		410	0.075	+	94		8–14
Perox membranes		1000	0.31		47		
Microsomes		320	0.6	+	690		
Perox/Mic		1.28	0.13		0.14		
Perox/Homogenate		3.42			0.39		
5. Appelkvist et al.	18						
Peroxisomes (recentrifuged)		70	0.041		60		1.5
Microsomes		540	0.19		4010		
Perox/Mic		0.13	0.22		0.015		
6. Fujiki et al.	13						
Peroxisomes			+			146	3.5
Microsomes			+ + + +				
Perox/Homogenate						0.175	

[a] Calculated from data of ref. 21.
[b] Clofibrate-treated rats.

reducible by an endogenous NADH–cyt b_5 reductase. The capacity of peroxisomes to incorporate purified b_5 was as large as that of microsomes when expressed per mole of phospholipid. Finally, in comparison to glucose-6-phosphatase activity, the peroxisomes appeared to have significant NADH–cytochrome *c* reductase (Table 2).

Huttinger and his co-workers purified peroxisomes from the livers of rats treated with clofibrate to induce proliferation of peroxisomes (11). NADH–cytochrome *c* reductase and cytochrome b_5 were present in the peroxisomes, and their specific activities increased 2.4–4 times when membranes (ghosts) were isolated by osmotic shock with pyrophosphate as described earlier. The specific activity of glucose-6-phosphatase decreased in the membranes relative to peroxisomes (although there was no positive control excluding enzyme inactivation as the cause). The cytochrome b_5 of intact peroxisomes could be efficiently reduced by 10 μM palmitoyl-CoA while that in the microsomes could not. After isolation of peroxisomal membranes, palmitoyl-CoA-dependent cytochrome b_5 reduction was less extensive and required the addition of soluble matrix material.

Peroxisomes isolated by Appelkvist et al. (18) by means of glutaraldehyde fixation and Percoll gradient centrifugation also appeared to contain cytochrome b_5 and NADH–cytochrome *c* reductase (Table 2). Their reported specific activity of glucose-6-phosphatase in microsomes was some 6–11 times greater than that observed by others (11, 21); any overestimation in this value would result in a corresponding underestimation of the microsomal contamination of peroxisomes. One must also consider the effect of glutaraldehyde on enzyme activities.

Surveying the data summarized in Table 2, one sees striking variations in the absolute values reported by different investigators. Peroxisomal cytochrome b_5 content ranges from 0.01 to 0.04 to 0.075 nmol/mg protein; part, but not all, of the difference can be accounted for by microsomal contamination. The difficulties in estimating contamination accurately are illustrated by the huge differences in reported specific activities of glucose-6-phosphatase in microsomes. This value is essential to the computation of contamination. In addition, the presence of some lysosomes in purified peroxisomal fractions may lead to an overestimation of microsomal contamination because lysosomal acid phosphatase has some activity toward glucose-6-phosphate (for which, however, a correction may be made—see ref. 9).

These data demonstrate the limits of preparative cell fractionation for evaluating the presence of a small proportion of an enzyme in a nonabundant cell structure. The analytical cell fractionation approach (23, 24) is better suited for this purpose. Specificially, if the distribution of

cytochrome b_5 and the reductases were compared with the distributions of marker enzymes in a continuous gradient, and a small peak of b_5 and the reductases coincided with the catalase peak, then one could conclude with much greater confidence that a portion of these activities was truly peroxisomal. This analytical approach allowed the demonstration that 7% of the liver's palmitoyl-CoA synthetase activity is in peroxisomes (25).

Despite these uncertainties, the data suggest that peroxisomal membranes probably contain a small portion of the liver's cytochrome b_5, NADH–cytochrome b_5 reductase, and NADH–cytochrome *c* reductase. In the case of cytochrome b_5, if we take the mean specific activities from Table 2 (0.04 nmol/mg in peroxisomes and 0.4 nmol/mg in microsomes) together with the abundance of peroxisomal and microsomal protein in liver (2.5 and 20% of homogenate protein, respectively), we conclude that per gram of liver, peroxisomes contain only 1/80 as much b_5 as microsomes. The most compelling evidence for this affirmative conclusion is the combination of the electron microscopic immunocytochemistry (19, 20) with the functional assays showing b_5 reduction by an endogenous peroxisomal reductase (20) and palmitoyl-CoA-dependent b_5 reduction in peroxisomes but not microsomes (26).

Another method that has been used to place enzymes in the membrane of peroxisomes is sensitivity to protease digestion. Mannaerts et al. (27) found that peroxisomal palmitoyl-CoA synthetase could be inactivated by pronase digestion under conditions where catalase remained latent. In addition, these authors reported that the ligase itself was not latent and required an external pool of CoA for activity. Taken together, these data indicate that the ligase is in the membrane, exposed to the outer, cytosolic side.

Bishop et al. (28) reported that acyl-CoA reductase (long-chain alochol-forming) is trypsin-sensitive in intact peroxisomes of rat brain. Two other enzymes involved in the synthesis of ether-linked glycerolipids [dihydroxyacetone phosphate (DHAP) acyltransferase and alkyl-DHAP synthetase] were trypsin-resistant unless the peroxisomes were permeabilized with Triton X-100. The DHAP acyltransferase had been shown to be a membrane-bound enzyme in guinea pig liver by its solubilization by detergents but not by 0.25 *M* KCl, osmotic shock, or freezing and thawing (29). The alkyl-DHAP synthetase is also thought to be a membrane-bound enzyme because of the need for detergent in its purification from Ehrlich ascites cell microsomes (30). Taken together, these data indicate that in rat brain DHAP acyltransferase and perhaps alkyl-DHAP synthetase are on the internal side of the peroxisomal membrane, whereas acyl-CoA reductase is on the external side.

Noguchi et al. (31) applied the pyrophosphate treatment to peroxisomes of fish and crustacean liver, which have an equilibrium density of 1.25 in sucrose, slightly denser than their mammalian counterparts. Upon recentrifugation in a sucrose gradient after pyrophosphate treatment, most of the enzymes assayed were soluble, but allantoicase exhibited a broad density distribution centered around a density of 1.17. In analogy with the results obtained with liver, Noguchi et al. (31) inferred that the allantoicase was bound to membranes. Presumably, as in liver these membranes were in fact ghosts, and therefore one cannot exlude the alternative that the allantoicase is in the residual content of the ghost.

2.3.2 Polypeptides

Fujiki et al. (13) analyzed by SDS-PAGE the integral membrane proteins in the unsealed membrane sheets (Figure 1*b*) prepared from rat liver peroxisomes by the carbonate procedure (Figure 2, lanes 2 and 6). These consist of three major polypeptides with molecular masses of 21,700, 67,700, and 69,700 daltons (the latter two not fully resolved in this experiment) plus a number of minor polypeptides. None of these appear to be glycoproteins on the basis of chromatography on lectin-Sepharose columns (32). The pattern of peroxisomal integral membrane proteins was compared with that of endoplasmic reticulum and mitochondrial membranes in adjacent lanes of a gradient slab gel (Figure 2). The differences far outweigh the similarities. These data demonstrate that although peroxisomal and endoplasmic reticulum membranes have some biochemical similarities (Table 2), on the whole they have highly dissimilar protein compositions. These data also confirm that there is minimal contamination of the peroxisomes by microsomes or mitochondria, which was inferred from the marker enzyme analyses (13) and the morphological appearance (Figure 1*a*). Moreover, the three major integral membrane polypeptides mentioned above were demonstrated to be truly peroxisomal by combining the power of SDS-PAGE in slab gels with analytical cell fractionation (13).

Peroxisomal membranes have a faint Coomassie-blue-stained band at the position of cytochrome b_5 (Figure 2). By itself this is not significant; in conjunction with the other results of Table 2 it supports the conclusion that peroxisomes contain small amounts of b_5.

Huttinger et al. (26) presented tracings of SDS-PAGE analyses of "peroxisomal membrane" preparations from normal and clofibrate-treated rats. They describe nine main protein bands, including a major 40,000-dalton polypeptide inducible by clofibrate in peroxisomes but not in mi-

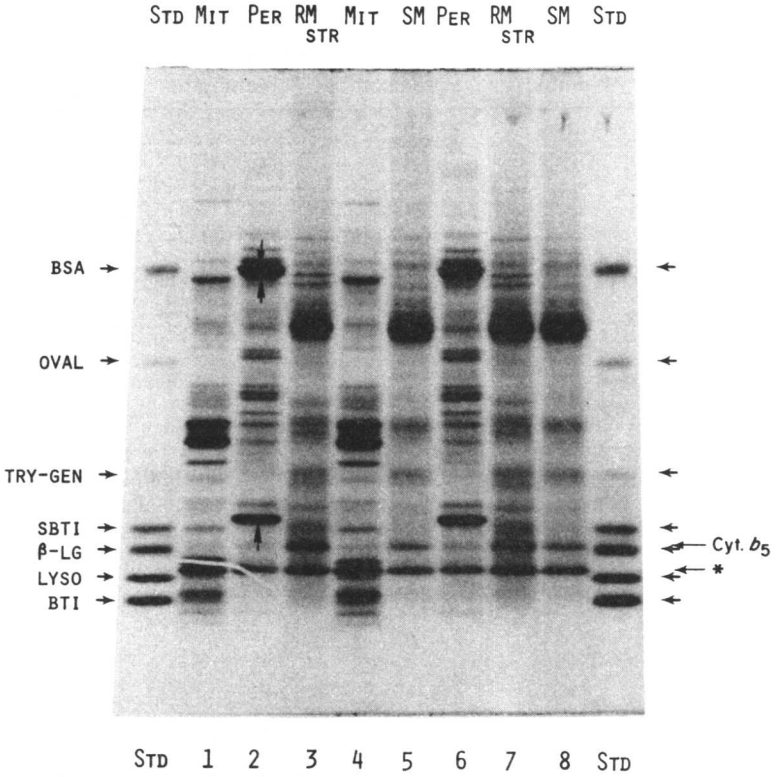

Figure 2 Polypeptide composition of rat liver organelle membranes prepared by the sodium carbonate procedure (14). Equal amounts of each type of membrane (50 μg protein) were analyzed by SDS-PAGE. Lanes 1 and 4, mitochondria; 2 and 6, peroxisomes; 3 and 7, rough microsomes stripped of ribosomes with pyrophosphate; 5 and 8, smooth microsomes. STD, molecular weight standards. Arrows in lane 2 indicate the major integral membrane proteins of the peroxisomes. Asterisk indicates a stained band visible in all the membranes. From ref. 13.

crosomes. The 40 kilodalton polypeptide is claimed to contain carbohydrate by periodic acid staining. This report contains no evaluation of the purity of the peroxisomes. It uses a somewhat different preparative procedure than that evaluated biochemically and morphologically in their later publication (11). No major polypeptide in this 40 kilodalton molecular mass region was found by Fujiki et al. (13) either in intact normal peroxisomes or in the membranes prepared therefrom by the carbonate procedure.

Table 3 Phospholipid Content (nmol/mg protein)

Source	Reference Number	Peroxisomes	Peroxisomal Membrane	Microsomes
Rat liver	10[a]	115		427
Rat liver	18[a]	147	733[b]	
Rat liver	13	36	204[c]	
Rat liver	33			470
			Glyoxysomal Membrane	
Castor bean endosperm	84		654[b]	1243
Castor bean endosperm	72		140[b]	704

[a] These data were given l y their authors in units of μg phospholipid/mg protein. They have been converted to nmol phospholipid/mg protein using an average molecular weight of 750.
[b] Ghosts.
[c] Carbonate-stripped open sheets

2.3.3 Phospholipids

Several authors have commented on the relatively low phospholipid/protein ratio of peroxisomes in comparison to other organelles (10, 13, 18), although the absolute values reported vary considerably (Table 3). The low phospholipid/protein ratio is due in part to the fact that the ratio of matrix protein to membrane protein is much higher in peroxisomes than in ER or in mitochondria (Table 1). After purification of peroxisomal membranes, the phospholipid/protein ratio increases 5–6 times (Table 3). In the case of carbonate-purified membranes, the ratio is 200 nmol phospholipid per mg protein, which is 43% of the value measured in total microsomes by Remacle (33) using similar methods.

The composition of peroxisomal phospholipids is similar to that of endoplasmic reticulum, consisting mainly of phosphatidylcholine and phosphatidylethanolamine, and perhaps some cholesterol; cardiolipin is absent (13). The fact that peroxisomal membranes are 10 times more refractory than lysosomal membranes to disruption by digitonin (1) also suggests that peroxisomal membranes contain little (if any) cholesterol.