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Studies on peroxisomal membranes

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The phospholipid/protein ratios of rat liver peroxisomes, mitochondria and microsomes were determined and found to be 257 ± 26, 232 ± 20 and 575 ± 20 nmol · mg^{-1}, respectively. After correction for the loss of soluble protein, a peroxisomal ratio of 153 nmol · mg^{-1} was calculated. Organelle fractions were treated with sodium carbonate, whereafter membrane fragments containing integral membrane proteins were pelleted. For the membrane fractions of peroxisomes, mitochondria and microsomes phospholipid/protein ratios of 1054 ± 103, 1180 ± 90 and 1050 ± 50 nmol · mg^{-1} were found, whereas 26 ± 2, 20 ± 2 and 49 ± 2% of the organelle protein was recovered in these membrane fractions, respectively. The phospholipid composition of the different organelle fractions were determined, but no large differences were obtained, except for cardiolipin that was found only in the mitochondrial fraction. After sodium carbonate treatment vitually all enzymatic activity of the enzymes tested was lost. Therefore Triton X-114 phase separation was used to obtain the peroxisomal membrane components. In this fraction 42.9 ± 3.5% of the protein and 90.2 ± 3.7% of the phospholipid was found. Enzymatic activity of two integral membrane proteins was recovered for over 90% in the membrane fraction, whereas activity of two matrix proteins was mainly found in the soluble fraction. Urate oxidase, the peroxisomal core protein, behaved differently and was recovered mainly with the membrane components. Recoveries of enzymatic activities after the Triton X-114 phase separation varied from 45 to 116%, and together with the good separation that was obtained between soluble proteins and integral membrane proteins this method provides a useful alternative for the isolation of membrane components.

Introduction

Peroxisomes are subcellular organelles bounded by a single membrane. They are widely spread in eukaryotic cells and appear to be ubiquitous in mammalian cells [1]. Peroxisomes carry out a diverse set of metabolic functions that varies with the cell type [2,3]. In mammalian cells among these are H_2O_2-metabolism [2], the β-oxidation of very long chain fatty acids [4,5] and the biosynthesis of ether phospholipids [6,7] and bile acids [8,9].

Recently, a new group of genetic diseases has been recognized in which peroxisomal functions are impaired [10]. These can be divided in diseases in which one (or several) peroxisomal enzyme(s) is (are) defected and diseases with a general impairment of peroxisomal functions [3,11]. In the latter case, among which Zellweger syndrome is the most prominent, at first no morphologically detectable peroxisomes were found in liver and kidney biopsies [3,11,12]. However, Santos et al. [13,14] showed with the use of antibodies against peroxisomal integral membrane proteins that in Zellweger fibroblasts 'peroxisomal ghosts' are present. These findings are in agreement with the idea, that in the case of a general impairment of peroxisomal functions there is a defect in the import machinery for matrix proteins [15,16]. From the above results it can be delineated that the peroxisomal membrane and peroxisomal integral membrane proteins have an important function in the import mechanism as has been shown already for protein import into endoplasmic reticulum [17] and mitochondria [18]. However, at present not much is known about factors that are involved in protein translocation across peroxisomal membranes [19,20].

Peroxisomal membranes have been studied in the past mainly by the group of Lazarow and co-workers [21]. Fujiki et al. [22] compared the polypeptide and phospholipid composition of membranes, isolated after sodium carbonate treatment [23] from rat liver peroxisomes (after Triton W-1339 injection), endoplasmic reticulum and mitochondria. They found only 12% of

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the total peroxisomal protein in the peroxisomal membrane. The phospholipid/protein ratio of intact peroxisomes was reported to be 36 and that of peroxisomal membranes 204 nmol · mg^{-1}. Microsomal membranes contained 53% of the total microsomal protein and had a phospholipid/protein ratio of 340 nmol · mg^{-1}, and mitochondrial membranes contained 21% of the total mitochondrial protein. Appelkvist et al. [24] isolated peroxisomes from rat liver after intraperitoneal injection of Triton W-1339 and found a phospholipid/protein ratio of 147 nmol · mg^{-1} for intact peroxisomes and a ratio of 733 nmol · mg^{-1} for isolated membranes, however, they used a different method [25] to isolate membranes. Donaldson et al. [26] isolated peroxisomes from rat liver after Triton-W1339 injection and found a phospholipid/protein ratio of 115 nmol · mg^{-1}. Finally, Crane et al. isolated peroxisomes from mouse liver without pretreating the animals and reported 22% of the protein to be integral [27]. Remarkably, however, upon treatment of peroxisomes and microsomes with sodium carbonate phospholipid/protein ratios of 729 ± 121 and 1012 ± 65 for the membrane fractions was found, respectively [28].

Although Fujiki et al. [23] report that the integral membrane proteins NADH–ferricyanide reductase and NADPH–cytochrome-*c* reductase remain active after carbonate treatment, to our knowledge no other reports on the recovery of active proteins after the sodium carbonate treatment have been made. Furthermore, several investigators have reported phospholipid/protein ratios for intact peroxisomes and isolated membranes that vary to a large extent. These considerations led us to reinvestigate the sodium carbonate treatment. First, to determine phospholipid/protein ratios in isolated peroxisomes and derived membranes. For comparison also mitochondrial and microsomal ratios and phospholipid composition of organelle membranes were determined. Secondly, to investigate the effects on enzymatic activities of several peroxisomal enzymes. Here, we also present an alternative method [29] to isolate integral membrane proteins applied to peroxisomes with the advantage of retaining enzymatic activity of most enzymes.

Materials and Methods

Materials

L-[U-^{14}C]Glycerol 3-phosphate, ammonium salt (171 mCi/mmol), [1-^{14}C]hexadecanol (55 mCi/mmol) and [9,10(n)-^{3}H]myristic acid (40–60 Ci/mmol) were obtained from Amersham (Buckinghamshire, U.K.). Metrizamide (2-(3-acetamido-5-*N*-methylacetamido-2,-4,6-triiodo-benzamido)-2-deoxy-D-glucose), analytical grade, was from Nyegaard and Co. AS (Oslo, Norway). Bovine serum albumin, essentially fatty acid free, acetoacetyl-CoA and sodium urate were from Sigma (St. Louis, U.S.A.). CoA, ATP and all enzymes were purchased from Boehringer (Mannheim, F.R.G.). Triton X-114 was from Aldrich (Beerse, Belgium) and all other chemicals were from Merck, (Darmstadt, F.R.G.) and were of standard laboratory grade.

Methods

Isolation. Peroxisomes were isolated as described before and were calculated to be at least 90% pure [30]. Mitochondria were isolated as described and were calculated to be 95% pure [31] and were a kind gift of R. Hovius. The microsomal fraction was obtained by pelleting the $25\,000 \times g$ supernatant of a 10% (w/v) rat liver homogenate for 60 min at $100\,000 \times g$.

Membrane isolation. The sodium carbonate procedure was carried out essentially as described by Fujiki et al. [23] except that membrane pelleting was carried out for 60 min at $134\,000 \times g$. For enzyme determination, directly after pelleting the pH of the supernatant fraction was adjusted to 7.4, whereas the pellet was resuspended (by sonication) in a buffer containing 150 mM NaCl and 10 mM Tris-HCl (pH 7.4). The Triton X-114 solubilization and phase separation was carried out as described by Bordier [29].

Lipid analysis. Lipids were extracted according to Bligh and Dyer [32] and lipid phosphorus was determined by the method of Rouser et al. [33]. The phospholipid composition of the organelle membranes was analysed according to Heymans et al. [34] using two-dimensional thin-layer chromatography (HPTLC plates silica gel 60, Merck, Darmstadt, F.R.G.), except that for development in the first direction chloroform/methanol/water (65 : 35 : 4, v/v) was used.

Assays. Enzymatic activities were determined as described previously [30]. Thiolase was determined as described [35] except that 50 μM acetoacetyl-CoA and 100 μM CoA were used. Urate oxidase was determined according to Leighton et al. [36], acyl-CoA ligase according to Wanders et al. [37] except that 60 nmol [9,10(n)-^{3}H]myristic acid(380 dpm/nmol) was used as a substrate. Protein was determined according to Lowry et al. [38] with bovine serum albumin as a standard and SDS-PAGE was performed according to Laemmli [39] and gels were stained with Coomassie brilliant blue.

Results and Discussion

Phospholipid/protein ratios

In Table I the phospholipid/protein ratios for intact organelles and carbonate pellets and supernatants are given. The values for mitochondria [26,3 1,40,41] and microsomes [26,41] are comparable to reported values. The peroxisomal value, however, differs markedly and is 2–7-fold higher than reported values [22,24,26]. One possible explanation for the discrepancies is the fragility of peroxisomes [30,36,42], resulting in the loss of soluble

TABLE I

Phospholipid/protein ratios of mitochondria, microsomes and peroxisomes and of the membrane and soluble fractions of these organelles upon sodium carbonate treatment

Organelle isolation, carbonate procedure and protein and phospholipid determinations were as described in Materials and Methods. Values ± (standard) deviation of mitochondria and microsomes are the mean of two experiments and for peroxisomes of three experiments.

	Mitochondria (nmol/mg)	Microsomes (nmol/mg)	Peroxisomes (nmol/mg)
Intact organelles	232 ± 20	575 ± 20	257 ± 26
Carbonate pellet	1180 ± 90	1050 ± 50	1054 ± 103
Carbonate supernatant	14 ± 3	20 ± 3	15 ± 2
	(%)	(%)	(%)
Membrane protein	20 ± 2	49 ± 2	26 ± 2

(matrix) proteins upon isolation of peroxisomes. Crane et al. [27] have also shown that upon dilution and resedimentation, a procedure that was used here, 23 ± 5% of the total protein is lost and Alexon et al. [42] reported a 35% loss of total (soluble) protein. When we calculate the phospholipid/protein ratio taking into account the contaminations with other organelles (4.5% mitochondria, 6.6% microsomes; [30]) and a 35% loss of total (soluble) proteins [42], a phospholipid/protein ratio of 153 nmol · mg^{-1} is obtained. This value is in reasonable agreement with the ratio's reported by Appelkvist et al. [24] and Donaldson et al. [26], but is much higher than the ratio found by Fujiki and coworkers [22]. The purity of the peroxisomal fraction used by Donaldson et al. has been disputed [22] and also the peroxisomal fraction isolated by Appelkvist may contain impurities, because only a 12-fold increase in catalase and a 14-fold increase in urate oxidase was reported. Fujiki et al. [22] used a very pure peroxisomal fraction, isolated according to Leighton et al. [36] who reported a 36-fold increase in catalase and a 50-fold increase in urate oxidase. These data indicate that, although not much, also soluble protein (catalase) was lost and from this it follows that the phospholipid/protein ratio for intact peroxisomes would even become lower than the 36 nmol · mg^{-1} reported by them. It is interesting to note that in the three investigations mentioned above, rats were preinjected with Triton W-1339 before isolating peroxisomes. Although it is assumed that this is taken up by lysosomes exclusively, in this way lowering the density, it may be that also peroxisomes are influenced perhaps resulting in a lower phospholipid/protein ratio.

When membrane fractions from the organelles were prepared [23], a similar phospholipid/protein ratio for the different organelle membranes was found. These findings are in reasonable agreement with the data of Crane et al. [28] for membranes from microsomes (1012 nmol · mg^{-1}) and peroxisomes (729 nmol · mg^{-1}) and also with those of Appelkvist et al. [24] for peroxisomal membranes (733 nmol · mg^{-1}). Again, however, the data of Fujiki et al. are much different, in that a ratio of 340 nmol · mg^{-1} for microsomal and 204 nmol · mg^{-1} for peroxisomal membranes was reported. Over 95% of the phospholipids were pelleted, which is more than the 83 ± 7% reported by Fujiki et al. [22]. The percentage of protein in membranes for mitochondria and microsomes (table I) resembles reported values of 21 and 53%, respectively [22]. The value for peroxisomal membranes is in reasonable agreement with the value (22%) reported by Crane et al. [27], but much higher than the 12% found by Fujiki et al. [22]. Also here, the loss of soluble proteins during isolation may have influenced the percentage of protein found in the membranes. When we take this into account and calculate the percentage of integral membrane proteins in intact peroxisomes, using the same assumptions as above, a value of 22% is calculated. Because 26% of the protein of isolated peroxisomes are membrane proteins, a 4-fold increase in the phospholipid/protein ratio would be expected in peroxisomal membranes when compared to the whole organelle, which is exactly what was found. The same calculation can be made and holds for the other organelles.

Phospholipid composition

In Table II the phospholipid composition of the different organelles is given. As expected, cardiolipin is only found in mitochondria [22,26,31,41] also indicating the purity of the different organelle fractions. The phospolipid composition of the peroxisomal membrane resembles much the mitochondrial membrane with respect to the phosphatidylethanolamine content and the microsomal membrane with respect to the phospha-

TABLE II

Phospholipid composition of mitochondrial, microsomal and peroxisomal membranes

Isolation of organelles and separation and determination of phospholipids were as described in Materials and Methods. Values are the mean of 2 experiments. Cardiolipin was corrected for carrying 2 phosphate groups. PE: phosphatidylethanolamine; pPE: plasmalogen phosphatidylethanolamine; PC: phosphatidylcholine; PS: phosphatidylserine; PI: phosphatidylinositol; Spm: sphingomyelin; CL: cardiolipin; x: unidentified.

Phospholipid	Phospholipid composition (%)		
	mitochondria	microsomes	peroxisomes
PE	29.0 ± 2.5	18.4 ± 1.1	27.5 ± 0.7
pPE	5.3 ± 0.2	2.3 ± 0.1	2.1 ± 0.2
PC	41.9 ± 0.9	55.4 ± 1.0	56.4 ± 0.2
PS	7.2 ± 1.1	7.4 ± 0.5	3.0 ± 1.1
PI	3.7 ± 0.1	10.5 ± 0.4	4.7 ± 0.8
Spm	1.6 ± 0.1	4.2 ± 0.7	3.7 ± 0.2
CL	7.6 ± 0.2	-	-
x	3.5 ± 0.2	0.3 ± 0.3	2.6 ± 0.3

tidylcholine content [22,26,28]. In all three organelles phosphatidylcholine and phosphatidylethanolamine are the main constituents, and only little sphingomyelin, phosphatidylserine, phosphatidylinositol and plasmalogen phosphatidylethanolamine is found.

Enzyme distribution and activity

To isolate peroxisomal membrane proteins, the phase separation method described by Bordier [29] making use of Triton X-114, was used. Table III gives the results of these experiments. In panel A the protein and phospholipid distribution are given, and it is shown that about 43% of the protein and 90% of the phospholipid is found in the Triton X-114 extract, designated pellet, indicating that hydrophobic proteins may be found in this fraction. The sucrose cushion between the two phases and the water phase contained 21 and 36%, respectively, of the protein and each about 5% of the phospholipid. Bordier [29] does not address to the presence of protein in the sucrose cushion between the phases, but shows in the miniprint that only about 80% of the bacteriorhodopsin is recovered, indicating that also part of this protein was lost in the sucrose cushion. However, recovery of haemoglobin was about 100%, thus suggesting that the nature of the protein influences its sedimentation into the sucrose cushion.

To investigate the distribution of proteins over the phases, enzymatic activities were determined in the three fractions. In panel B of Table III the results are given. For these enzyme distribution studies the activities measured in the sucrose cushion were added to those recovered in the water phase and collectively presented as activity in the supernatant fraction, because it was found that mainly soluble proteins were present in the cushion. Catalase and thiolase are both soluble proteins found in the peroxisomal matrix [16,42]. Catalase activity is almost completely found in the supernatant fraction, where also most of the thiolase activity (78%) is present. The last two columns of panel B compare the recoveries of the Triton X-114 method to the sodium carbonate method. Both enzymes showed a very good recovery with the first method, but almost no activity remained after the carbonate extraction.

Acyl-CoA ligase [42,43] and alkyl-DHAP synthase [30,44] are both integral membrane proteins. Both enzymes were found for over 90% in the Triton X-114 pellet, indicating that indeed integral membrane proteins distribute, together with the phospholipids, in this fraction. These data also show that the sucrose cushion contained little if any hydrophobic proteins (and only 5% of the phospholipid). Again, the recovery of the acyl-CoA ligase was good with the triton method, whereas the alkyl-DHAP synthase activity is lost for about 50%. Both activities were lost completely, however, upon sodium carbonate treatment. The integral membrane protein dihydroxyacetone-phosphate acyltransferase was also tested, but with both methods the activity was lost completely. With respect to this last protein, however, it is well known that this enzyme is extremely labile in the presence of detergents [30].

Finally, the distribution of urate oxidase was tested. Urate oxidase is an abundant peroxisomal protein and is the major if not only constituent of the crystalloid core of rat liver peroxisomes [16,42,45]. It was expected that this protein might behave differently from other matrix proteins, because it is not solubilized by detergents [24,42]. The data indeed prove this. Using the triton method, 97% of the activity was found in the triton pellet and the recovery of activity was about 76%. In this way urate oxidase behaved differently in the triton method, when compared to the sodium carbonate method where it was found almost completely in the

TABLE III

Distribution of peroxisomal protein, phospholipid and enzymatic activities over the membrane and soluble fractions upon Triton X-114 phase separation

Triton X- 114 extraction (1 mg peroxisomal protein), carbonate treatment, enzyme assays and determination of protein and phospholipid were as described in Materials and Methods. In panel B the activities in the sucrose cushion and the water phase are combined and given as the supernatant fraction. Total activities are given in $\mu mol \cdot min^{-1}$ (catalase, thiolase and urate oxidase), $nmol \cdot min^{-1}$ (acyl-CoA ligase) and $pmol \cdot min^{-1}$ (alkyl-DHAP synthase). Values are given as the mean ± (standard) deviation and the number of determinations(n) is given in brackets. (A) Protein and phospholipid distibution after Triton X-114 extraction. (B) Effects on enzyme distribution and activities. n.d., not detectable.

A	Pellet (%)	Sucrose (%)	Water (%)	Recovery (%)
Protein(5)	42.9 ± 3.5	21.1 ± 3.0	36.0 ± 3.3	90 ± 25
Phospholipid(3)	90.2 ± 3.7	5.0 ± 2.7	4.9 ± 1.1	78 ± 5

B	Pellet		Supernatant		Recovery	
Enzymes	total activity	%	total activity	%	Triton	carbonate
Catalase(3)	0.35 ± 0.18	5.1	6.56 ± 1.04	94.9	91 ± 3	n.d.
Thiolase(3)	1.59 ± 0.22	21.4	5.84 ± 0.23	78.6	116 ± 16	4
Acyl-CoA synthetase(3)	15.1 ± 10.5	91.3	1.44 ± 0.36	8.7	82 ± 63	n.d.
Alkyl-DHAP synthase(2)	48.30 ± 4.20	95.0	2.53 ± 1.15	5.0	45 ± 5	n.d.
Urate oxidase(3)	0.39 ± 0.13	92.0	0.03 ± 0.01	8.0	76 ± 10	n.d.

supernatant [22]. However, again no activity remained after the carbonate precipitation. Preliminary experiments indicate (data not shown) that it should not be difficult to remove the urate oxidase before the phase separation is induced by increasing the temperature, because it has been shown that the crystalloid core is not solubilized by detergents and retains its density and therefore is easy to pellet [24,42]. Moreover, 10% of the total peroxisomal protein is urate oxidase [36,45,46]

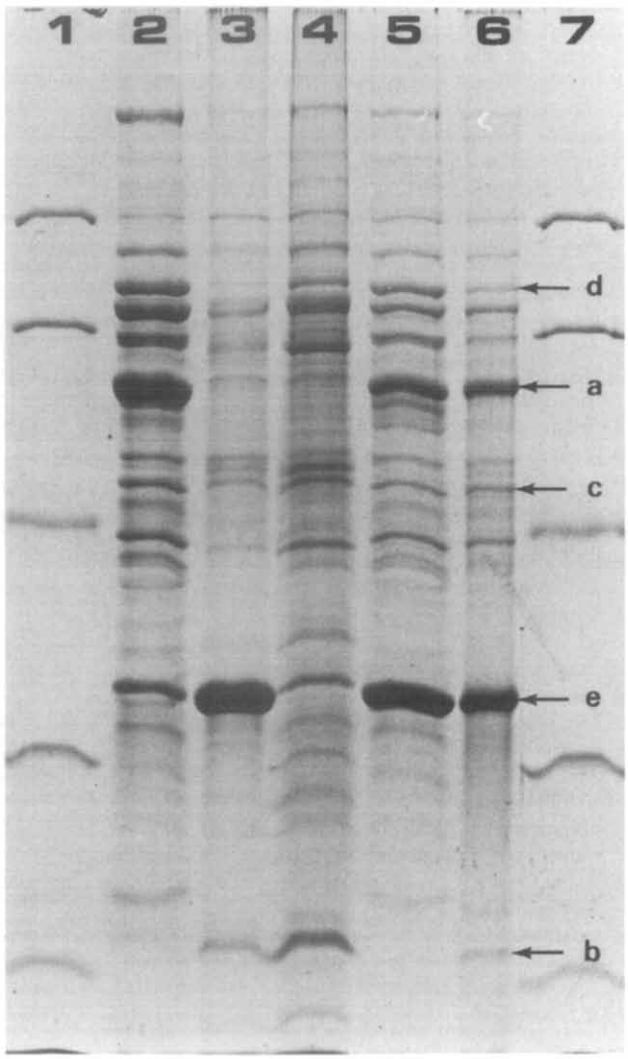

Fig. 1. Comparison of the protein patterns of the peroxisomal membrane and soluble fractions alter Triton X-114 and sodium carbonate treatment. Purified peroxisomes were treated with Triton X-114 [29] or sodium carbonate [23] as described to obtain the membrane and soluble fractions. Equal amounts of protein (20 μg) were applied to a 10% SDS-polyacrylamide gel and the gel was stained with Coomassie brilliant blue. Molecular weight markers were phosphorylase B (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45.0 kDa), carbonic anhydrase (31.0 kDa) and soybean trypsin inhibitor (21.5 kDa). 1, molecular weight markers; 2, Triton X-114 water phase (soluble); 3, Triton X-114 membrane fraction (pellet); 4, sodium carbonate membrane fraction (pellet); 5, sodium carbonate soluble fraction (supernatant); 6, peroxisomes; 7, molecular weight markers. Letters used to designate proteins are described in the text.

from which follows that only 32.9% of the total protein is found in the membrane fraction. This is still higher than the 26% we found with the carbonate treatment which may be explained by the observation that soluble proteins like thiolase and adhering membrane proteins also partitioned partially in the the triton phase.

Gel patterns

In Fig. 1 the peroxisomal protein patterns of membrane and soluble fractions after applying both methods are compared. Both methods give a clearly distinct pattern for soluble and integral membrane proteins. On the other hand both the soluble and integral fractions prepared by the different methods show large similarities. For example protein a (compare Fig. 1) is with both methods almost completely recovered in the supernatant fraction and protein b (22 kDa) in the integral membrane protein fraction. Not all proteins show an absolute distribution as can be seen with protein c, which is found with both methods in the soluble as well as in the pellet fractions. Another explanation for this observation is, of course, that this band reflects two proteins with the same molecular weight. Finally, there are also differences between the two methods. Protein d is found in both fractions using the carbonate treatment, whereas with the triton method it is almost completely soluble. Another exception is the very heavy stained band around 35 kDa (protein e), which is found mainly in the pellet after the triton phase separation and in the supernatant after the sodium carbonate method. We believe that this is urate oxidase, which as is shown in Table III, behaves differently because it is the cristalloid core protein.

Conclusions

The phospholipid/protein ratio of peroxisomes has been reported to be low when compared to other organelles. We find a much higher ratio of 257 ± 26 nmol $\cdot$ mg^{-1}. When we calculate the ratio including the loss of soluble protein, which has not been done by others, also a relatively low ratio of 153 nmol $\cdot$ mg^{-1} is obtained. Using the sodium carbonate treatment, we obtained a phospholipid/protein ratio for all membranes of around 1050 nmol $\cdot$ mg^{-1}. Our data and also those obtained by others indicate that the ratio's found by Fujiki et al. [22], presented in the paper in which the carbonate treatment was introduced, are too low. These data, therefore, further indicate that the phospholipid/protein ratio in peroxisomal membranes is not uniquely low but comparable to that of other biomembranes. An important drawback of the carbonate precipitation is the loss of enzymatic activity. Therefore, the Triton X-114 phase separation method [29] was used to study the peroxisomal membrane proteins. A good separation between soluble and integral membrane proteins was

obtained, with the large advantage that most enzymes retained their enzymatic activity. The method is probably not as exclusive as the carbonate method in separating integral and peripheral membrane proteins and also part of the soluble proteins, like thiolase, may be found in the triton phase, resulting in a higher percentage of protein found in the membrane fraction. More importantly we believe, however, is that the integral membrane proteins we tested were found completely in the triton phase and, with exception of the DHAP-AT, were partially or fully active. This is an important observation which can be used in further studies on integral membrane proteins.

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