

Biogenesis of Peroxisomes: Isolation and Characterization of Two Distinct Peroxisomal Populations from Normal and Regenerating Rat Liver

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Abstract. According to Poole et al. (1970. *J. Cell Biol.* 45:408–415), newly synthesized peroxisomal proteins are incorporated uniformly into peroxisomes (PO) of different size classes, suggesting that rat hepatic PO form a homogeneous population. There is however increasing cytochemical and biochemical evidence that PO in rat liver are heterogenous, undergoing significant modulations in shape and size in process of PO morphogenesis (Yamamoto and Fahimi, 1987. *J. Cell Biol.* 105:713–722). In the present study, the kinetics of incorporation of newly synthesized proteins into distinct PO-subpopulations have been studied using short-term in vivo labeling (5–90 min). Two distinct “heavy” and “light” crude PO fractions were prepared by differential pelleting from normal and regenerating liver, and highly purified PO were subsequently isolated by density-dependent metrizamide gradient centrifugation according to Völkl and Fahimi

(1985. *Eur. J. Biochem.* 149:257–265). The peroxisomal fractions banded at 1.20 and 1.24 $g \times cm^{-3}$. They differed in their mean diameters and form-factors and particularly in respect to the activity of β -oxidation enzymes which was higher in the “light” PO. Whereas the “light” PO exhibited a single immunoreactive band with the antibody to the 70-kD peroxisomal membrane protein the “heavy” PO contained an additional (68 kD) band. In pulse-labeling experiments “light” PO showed clearly a higher initial rate of incorporation than the “heavy” PO. The relative specific activity in the “heavy” PO fraction, however increased progressively reaching that of “light” PO by 90 min. These observations provide evidence for the existence of different PO populations in rat liver which differ in their morphological and biochemical properties as well as in their rates of incorporation of new proteins.

IT is well established that new peroxisomes (PO)¹ form by division from preexisting ones, and most PO proteins, including the membrane proteins are synthesized on free ribosomes and incorporated posttranslationally into the organelle (for a review see Lazarow and Fujiki, 1985). Moreover, it is generally assumed that peroxisomes form structurally and functionally a uniform intracellular compartment (Lazarow et al., 1980; Borst, 1986). Indeed, in almost all original studies carried out in rat liver no differences in enzyme distribution were observed as a function of the size and density of the isolated organelles (Beaufay et al., 1964; Leighton et al., 1969; Poole et al., 1970; Lazarow and de Duve, 1973). More importantly, Poole et al. (1970) who studied the growth of peroxisomes as a function of age in a time span of 3 h to 1 wk, found no evidence of maturation of the organelle. In recent years however, evidence has been

accumulating that PO differ not only morphologically and functionally in different cell types (Böck et al., 1980; Lazarow, 1988; van den Bosch et al., 1992) but also within one and the same cell. This is exemplified by the heterogeneous distribution of various cyto- and immunocytochemically detected peroxisomal oxidases and catalase in rat and human hepatocytes (Le Hir and Dubach, 1980; Angermüller and Fahimi, 1986, 1988; Fahimi et al., 1982; Baumgart et al., 1989; Roels and Cornelis, 1989), and by the isolation of peroxisome subpopulations with divergent equilibrium densities and enzyme compositions (Flatmark et al., 1980; Goglia et al., 1990; Klucis et al., 1991; Aikawa et al., 1991). Finally, temporal alterations of the peroxisomal compartment were described in regenerating rat liver with the formation of a peroxisomal reticulum at the early stages (Yamamoto and Fahimi, 1987b) and its replacement by single mature peroxisomes at later intervals (Yamamoto and Fahimi, 1987a) demonstrating the dynamic course of peroxisomal biogenesis. In view of these recent observations, we reinvestigated by pulse-labeling the incorporation of newly synthesized proteins into subpopulations of PO. In our initial experiments we found, by using shorter pulse labeling intervals

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1. *Abbreviations used in the paper:* PMP, peroxisomal membrane protein; PO, peroxisome; RCF, relative centrifugal force; RSA, relative specific activity.

of 30 min instead of 3 h, as used by Poole et al. (1970), that newly synthesized proteins were incorporated preferentially into smaller-sized PO (abstracts; Lüers et al., 1991. *Eur. J. Cell Biol.* [Suppl.]32:15; and Lüers et al., 1992. *Mol. Biol. Cell.* 3:182a). A similar observation was reported by Heinemann and Just (1992) in respect to the import of newly synthesized acyl-CoA oxidase into PO with a lower equilibrium density than the regular ones.

In the present study we have devised a method for the isolation of two distinct subpopulations of PO with differing sizes and densities from rat liver which is based on our earlier approach using metrizamide gradient centrifugation (Völkl and Fahimi, 1975). In addition, *in vivo* pulse studies with [³⁵S]methionine have been carried out to assess the exact role of those PO-subpopulations in the uptake and transport of new peroxisomal proteins. The results show that the two PO-subpopulations which differ in several respects such as in membrane protein composition and in activity of fatty acid β -oxidation enzymes exhibit also significant differences in the rate of incorporation of newly synthesized proteins.

Materials and Methods

Partial Hepatectomy

Normal female Wistar rats weighing \sim 220 g and kept on a normal laboratory diet and water *ad libitum* were used. They were housed in a room with a light and dark cycle of 12 h and were subjected after an overnight fast to partial hepatectomy. Under chloralhydrate anesthesia, the median and left lateral lobes of the liver (\sim two thirds) were removed using a modification of the method of Higgins and Anderson (1931). More than 90% of rats survived the operation. After hepatectomy, animals were housed individually with free access only to water. All animals were killed between 8 and 9 a.m. and the timing of partial hepatectomy was adjusted accordingly.

Cell Fractionation and Isolation of Peroxisomes

Main fractions and highly purified peroxisomes were prepared as outlined in Fig. 1, extending the fractionation scheme described earlier (Völkl and Fahimi, 1985). Briefly, liver tissue was minced and homogenized in (1g/5ml) ice-cold homogenization buffer: 5 mM MOPS, pH 7.4, 250 mM sucrose, 1 mM EDTA, 0.1% ethanol, 0.2 mM PMSF, 1 mM ϵ -aminocaproic acid and 0.2 mM DTT.

Total homogenates were subfractionated by differential pelleting, yielding mitochondrial, microsomal and cytosolic fractions in addition to an enriched "light" and another "heavy" peroxisomal fraction. The latter two fractions were subjected to density gradient centrifugation on preformed metrizamide gradients (1.08 – $1.27 \text{ g} \times \text{cm}^{-3}$) using a Beckman VTi 50 vertical type rotor in a Beckman L5-65 B ultracentrifuge (Beckman Instruments, Inc., Fullerton, CA). Gradients were prepared by adjusting the density of metrizamide in gradient buffer (homogenization buffer without sucrose) to 1.12, 1.155, 1.19, 1.225 and $1.26 \text{ g} \times \text{cm}^{-3}$. This discontinuous gradient was immediately frozen in liquid N₂ and stored at -20°C . Before use, the frozen gradients were thawed at room temperature as quickly as possible (\sim 30 min), giving rise to an exponentially shaped gradient profile. Spun at an integrated relative centrifugal force (RCF) = $1.25 \times 10^6 \text{ g} \times \text{min}$ ($39,000 \text{ g}_{\text{max}}$) highly purified "light" POs banded at $1.208 \text{ g} \times \text{cm}^{-3}$, whereas the "heavy" POs were concentrated in the density range of $1.24 \text{ g} \times \text{cm}^{-3}$. POs aspirated by means of a syringe were further subfractionated to obtain a matrix as well as a membrane fraction using the carbonate buffer procedure (Fujiki et al., 1982). All manipulations were carried out in a cold room (4 – 5°C).

Biochemical Analyses

Standard procedures were employed for the determination of peroxisomal enzymes (for references see Völkl and Fahimi, 1985), and protein was determined by the Lowry method (1951) using BSA as a standard. All assays were run with a recording spectrophotometer (Kontron, Uvikon 810, Munich, FRG).

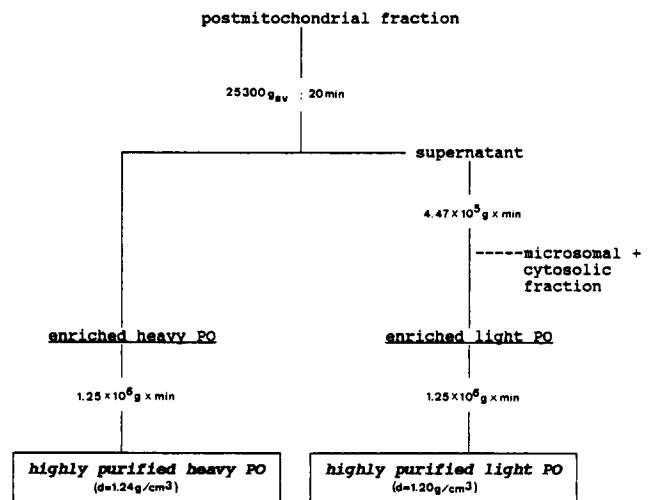


Figure 1. Diagrammatic presentation of the fractionation scheme for the isolation of "heavy" and "light" PO from rat liver. The details are given in Materials and Methods.

SDS-PAGE

SDS-PAGE was carried out using a polyacrylamide resolving gel of 10–15% ($18 \times 31 \times 0.1 \text{ cm}$; linear gradient) run at constant voltage for 1,800 V \times h. Polypeptides were alkylated before to electrophoresis by means of 30 mM iodoacetamide. The amount of protein loaded per lane of "heavy" and "light" PO and corresponding membrane preparations are indicated in Fig. 6. The gel was stained with Coomassie brilliant blue R 250 for visualization.

Immunoblotting

Minigels ($8.5 \times 5 \times 0.1 \text{ cm}$, 10% resolving and 5% stacking gel) were used for SDS-PAGE, and electrotransfer to nitrocellulose sheets was done at 100 mA by semidry blotting (Kyhse-Andersen, 1984). Polyclonal monospecific antibodies raised in rabbits were employed for immunocomplexing and protein A-gold for visualization as described recently (Beier et al., 1988).

Preparation of Antibodies

The preparation of antibodies and assessment of their monospecificities to peroxisomal catalase, urate oxidase and the 70-kD PO-membrane protein (PMP 70) has been reported recently (Beier et al., 1988; Baumgart et al., 1989). The total IgG-fractions were adjusted to \sim 30 mg protein per ml and were diluted about 100-fold in 10% newborn calf serum (NCS)/PBS/0.1% Tween 20 (NCS/PBS/T) for immune overlay.

In Vivo "Pulse Labeling" of Newly Synthesized Proteins

Under chloralhydrate anesthesia each animal was injected with 10 μCi L-[³⁵S]methionine (37 TBq/mmol) diluted in 0.2 ml PBS into the portal vein. Livers were removed after 5, 15, 30, and 90 min, respectively, and homogenized in ice-cold homogenization buffer supplemented with 0.3 mM cycloheximide as well as 2 mM "cold" methionine. Main fractions were prepared and highly purified "light" and "heavy" POs were isolated as described above. POs were further subfractionated according to Fujiki et al. (1982) to yield matrix and membrane subfractions. Soluble (main fractions) and TCA precipitable radioactivities were determined in a liquid scintillation counter (LS 6000 SC, Beckman, Munich, Germany) using the "Ready Safe" cocktail of the same supplier. The contribution of radioactivity due to microsomal contamination in the PO-fraction was taken into consideration and was calculated as follows: esterase activities in PO-fractions and appropriate total homogenates were determined and used to calculate the amount of microsomal protein (in μg) contaminating PO fractions. The corresponding radioactivity was calculated from that of the crude microsomal fraction and was subtracted from the radioactivity of the PO fraction.

Electron Microscopy and Morphometry of Highly Purified Peroxisomes

For morphological studies "light" and "heavy" peroxisomal fractions obtained from both control and hepatectomized animals were fixed, embedded and processed as described (Völkl and Fahimi, 1985). Ultrathin sections of 50 nm thickness were examined in a Philips 301 electron microscope.

Morphometry of Peroxisomes

The surface areas of peroxisomal profiles were determined by means of the television-based Texture Analysis System (TAS) of Leitz (Wetzlar, FRG) (Beier and Fahimi, 1992). The data obtained were used to compute the diameter of the area-equivalent circle of the peroxisomal profiles. Moreover, formfactors (FF) were calculated by the formula

$$FF = \frac{4 \pi \times A_p}{P^2},$$

whereby A_p is the single profile area and P is the perimeter of the peroxisomal profile. The formfactor (FF) is 1 for an ideal circle and decreases with increasing deviation from the circle form.

Results

Isolation of "Light and Heavy Peroxisomes"

Employing the fractionation scheme outlined in Fig. 1, the subfractionation of homogenates from control as well as

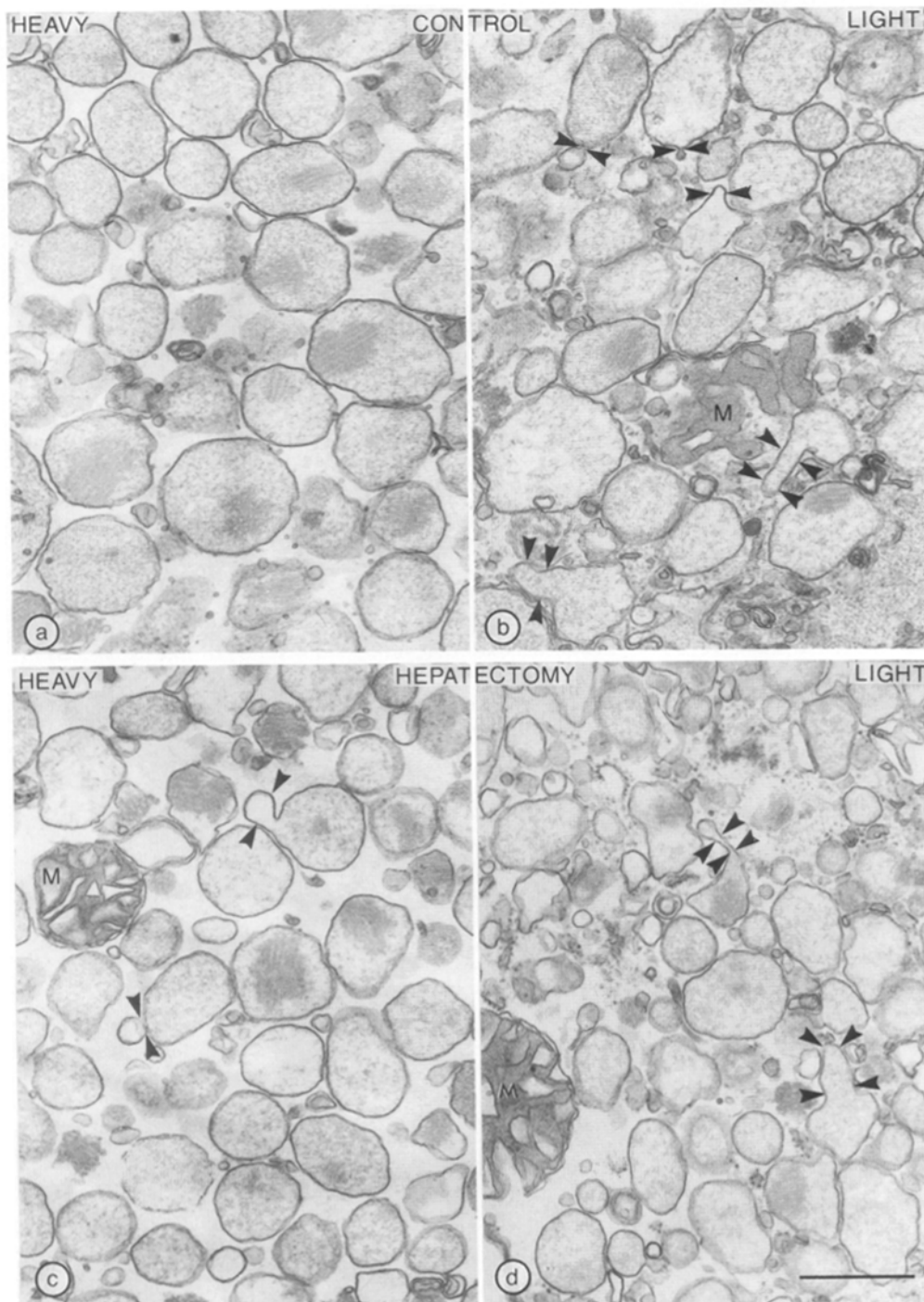


Figure 2. Ultrastructural appearance of isolated "heavy" (a and c) and "light" (b and d) PO from control (a and b) and regenerating livers (c and d). Note the presence of budding PO particularly in the "light" PO (arrowheads) of both control and regenerating livers. M, mitochondria. Bar, 0.5 μ m.

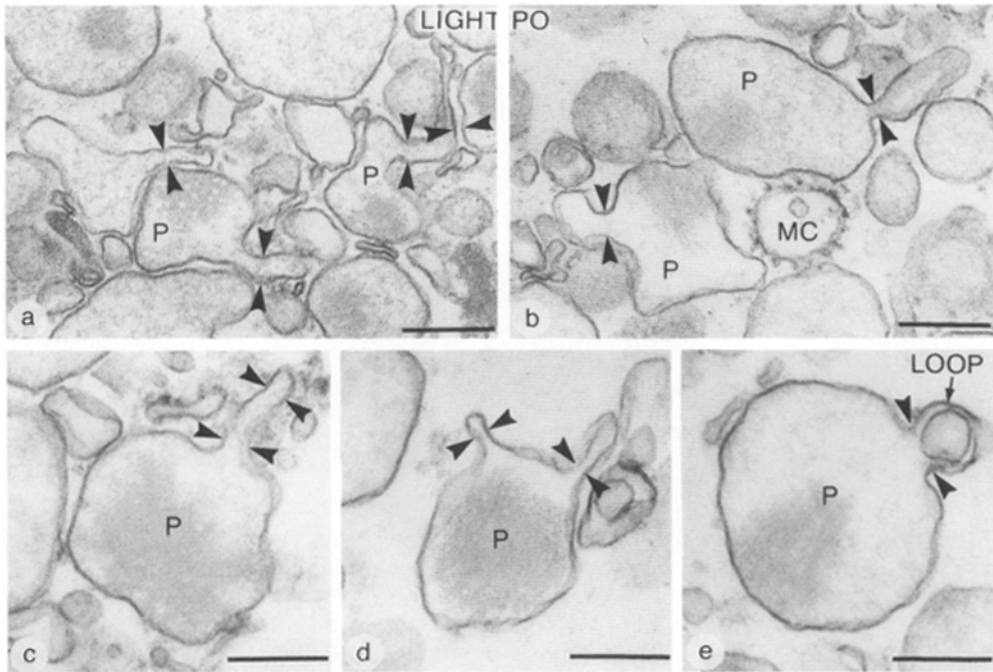


Figure 3. Higher power views of isolated "light" PO from regenerating rat liver showing tubular membrane extensions and buds (arrowheads). (e) A peroxisome (P) with a double-membrane loop (LOOP) is depicted. (MC) Microsome. Bars, 0.2 μm .

regenerating liver by differential and density gradient centrifugation resulted in two peroxisomal fractions. The first one banding at a density of $1.2407 \text{ g} \times \text{cm}^{-3}$ and designated the "heavy" peroxisomal fraction comprises POs characterized morphologically and biochemically in our earlier studies (Völkl and Fahimi, 1985; Lüers et al., 1990). The second one referred to furtheron as the "light" peroxisomal fraction was recovered from the exponential gradient at $1.2084 \text{ g} \times \text{cm}^{-3}$. Those "light" peroxisomes have not yet been characterized in detail and will be treated in this study.

Morphological Characterization of Isolated Peroxisomes

Fig. 2 shows the ultrastructural appearance of "heavy" (Fig. 2, a and c) and "light" (Fig. 2, b and d) PO fractions isolated from control (Fig. 2, a and b) and regenerating (Fig. 2, c and

d) rat livers. Evidently, all fractions consist almost exclusively of POs which appear well preserved with slight contamination by mitochondria, which is least prominent in the "heavy" POs of control rats (Fig. 2 a). A distinct limiting membrane without any disruptions surrounds the fine granular matrix of most particles. Most crystalline cores are found in the matrix of POs with only a few rare instances of extrusion. Fig. 2 also documents that "light" POs are consistently smaller in size and more irregular in shape than the "heavy" ones (Fig. 2, a and c).

Some of the typical morphological features of the "light" peroxisomal fraction are depicted in Fig. 3, which shows membraneous buds, invaginations and tail-like extensions in direct continuity with the peroxisomal membranes. Such structures were most prominent and numerous in the "light" PO fractions from the regenerating rat liver.

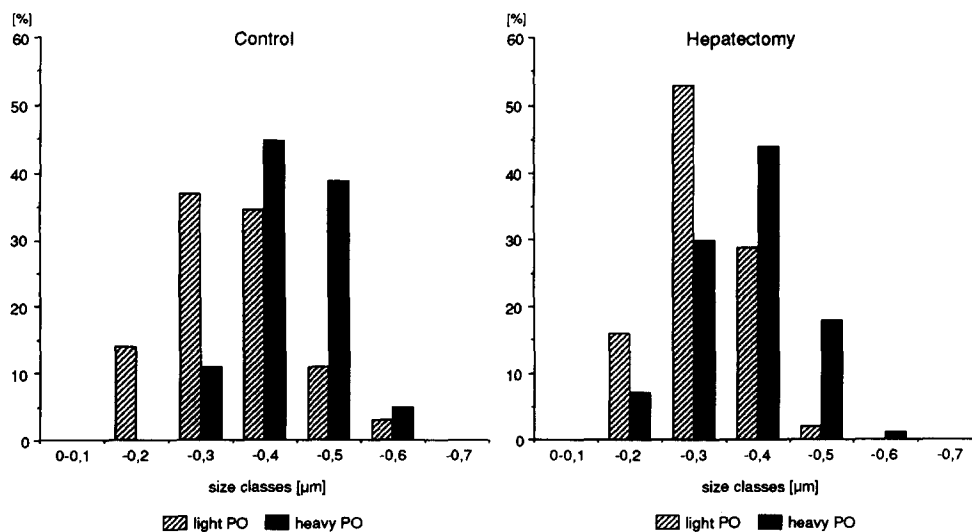


Figure 4. Mean diameters of isolated "light" and "heavy" PO ($n = 100$) from control and regenerating liver determined by morphometry as outlined in Materials and Methods. Note the higher frequency of smaller PO in the "light" PO fractions and their increase after partial hepatectomy.

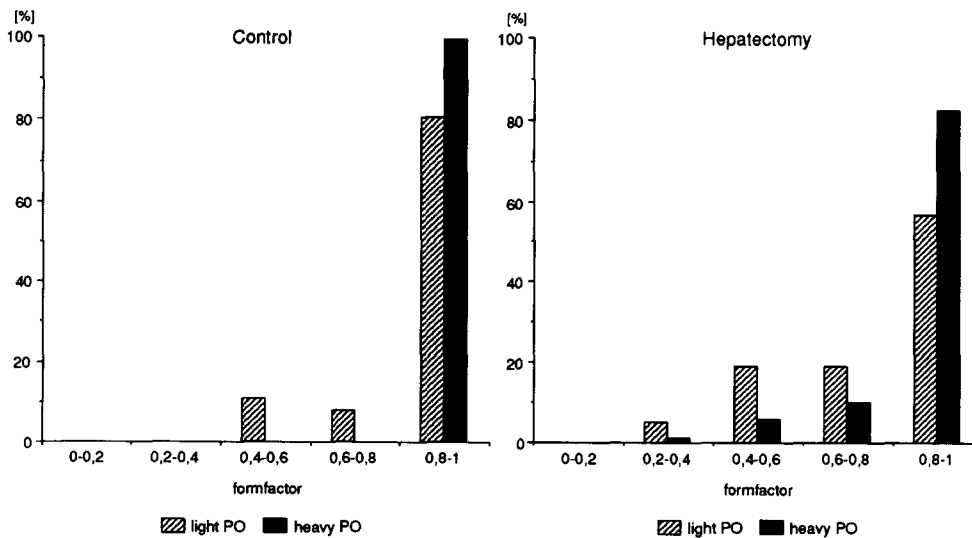


Figure 5. The formfactor of "light" and "heavy" PO ($n = 100$) from control and regenerating livers calculated as described in Materials and Methods. Note the higher frequency of particles deviating from the circle form in the "light" PO fraction and their relative increase after partial hepatectomy.

Morphometry of Isolated Peroxisomes

The differences observed in size and shape of isolated POs are substantiated by morphometric data (Figs. 4 and 5; Table I). Thus, there are distinct variations in diameter and size distribution between "light" and "heavy" particles from both control and regenerating livers with the ranking in mean diameter as listed in Table I. Alterations in the formfactor (FF) shown in Fig. 5 parallel the variations in size distribution. Again, "light" POs of the regenerating liver exhibit the most conspicuous variation in shape with most deviation from the circle form (Table I).

Properties of Purified "Light" Peroxisomes

Specific Activities of Marker Enzymes. The main enzymatic properties of the "light" peroxisomal fraction are listed in Table II, compared to those of the "heavy" peroxisomal fraction. In preparations of control animals the specific activities of catalase and uricase are quite the same in both fractions, whereas those of acyl-CoA oxidase and trifunctional protein are significantly higher in the "light" POs. In regenerating liver, the "heavy" POs are essentially unaltered in comparison to controls, but the activities in "light" PO of all enzymes particularly those of the β -oxidation system are markedly reduced.

Integrity of Isolated Peroxisomes. To assess the integrity of isolated "light" POs, the percentage recovery from post-nuclear fraction of soluble catalase and insoluble uricase in purified "light" and "heavy" POs of controls were compared

Table I. Diameter and Formfactor of Peroxisomes Isolated from Control and Regenerating Rat Liver

	Diameter nm	Formfactor
Heavy PO-control	387 \pm 7.15	0.930
Light PO-control	301 \pm 8.94	0.890
Heavy PO-PH	321 \pm 8.72	0.911
Light PO-PH	268 \pm 0.80	0.808

Results given are mean values \pm SEM with 100 PO evaluated per fraction. PH, partial hepatectomy (32 h).

(Table III). The data indicate, that both enzymes were recovered in each of the fractions at quite the same rates, i.e., catalase: 3.7 and 0.9%; uricase: 3.7 and 1.2%. These results suggest that during the isolation procedure, "light" and "heavy" POs were affected to the same extent by mechanical stress due to the fractionation procedure. This implies that the fractions did not differ significantly in their preservation state and that "light" POs did not arise by damage or fragmentation from intact "heavy" POs.

SDS-PAGE and Immunoblotting of Peroxisomal Fractions. In SDS-PAGE, no significant qualitative differences were found between the "light" and "heavy" peroxisomes except for a few bands due to apparent microsomal contamination as indicated in Fig. 6. The peroxisomal membrane subfraction however, exhibited some differences which were best visualized in Western blots. Fig. 7 shows immunoblots of "light" and "heavy" POs from control and regenerating

Table II. Enzyme Activities (U/mg) of "Light" and "Heavy" Peroxisomes from Control and Regenerating Rat Liver (32 h PH)

	Heavy PO		Light PO	
	Control	Regeneration	Control	Regeneration
Peroxisomal enzymes				
Catalase	9.24	9.73	7.31	4.88
Urate oxidase	0.065	0.068	0.073	0.050
Acyl-CoA oxidase	4.17	4.57	5.31	2.69
Trifunctional protein*	1.91	1.64	2.73	0.78
Microsomal and mitochondrial enzymes in PO fractions				
Esterase ^{1,2}	0.156	0.087	0.754	0.325
Cytochrome <i>c</i> oxidase ^{1,2}	0.033	0.014	0.0423	0.021

The results are of one representative experiment.

* β -enoyl-CoA hydratase assayed according to Osumi and Hashimoto (1979).

^{1,2} Enzyme activities (U/mg) in total homogenates, 1 in control: esterase 1.235; cytochrome *c* oxidase 0.089 2 in regenerating liver: esterase 0.610; cytochrome *c* oxidase 0.072.

Table III. Rates of Recovery ($U_{PO} \times \Sigma U^{-1}$) of Catalase and Urate Oxidase in Highly Purified "Light" and "Heavy" Peroxisomes of Control Liver in Relation to Total Homogenate and Appropriate Enriched Peroxisomal Fractions

Catalase	Percentage recovery	
	Total homogenate %	Enriched PO %
Total homogenate	100% = 440.7 U	
Enriched heavy PO	30.0	100
Purified heavy PO	3.7	12
Enriched light PO	14.5	100
Purified light PO	0.9	6
Urate oxidase		
Total homogenate	100% = 3.107 U	
Enriched heavy PO	33.0	100
Purified heavy PO	3.7	11
Enriched light PO	23.0	100
Purified light PO	1.2	5

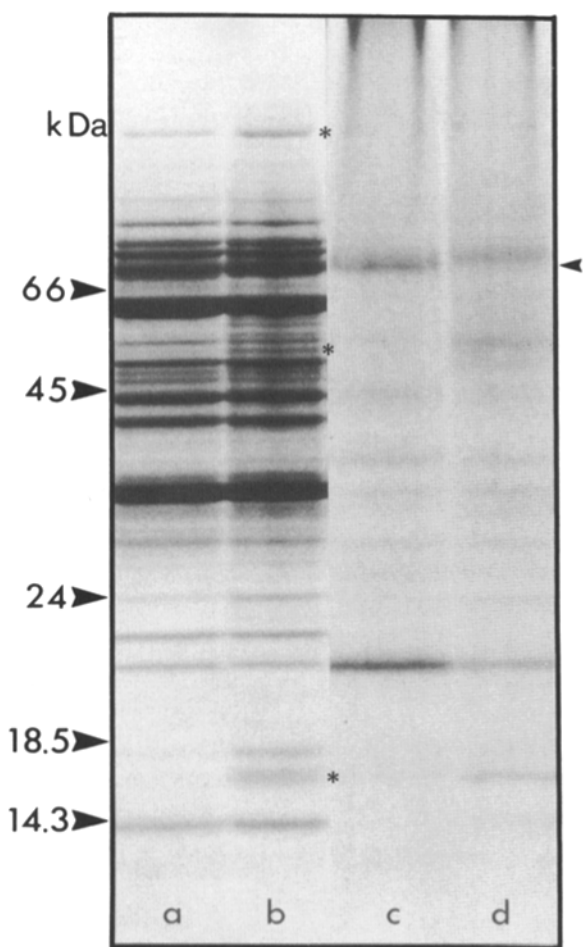


Figure 6. SDS-PAGE of total and membrane fractions of "heavy" (a,c) and "light" (b,d) POs from control liver. A 10–15% resolving gel was used and μg of protein loaded per lane were: (a) 44.9; (b) 62.7; (c) 36.8; and (d) 42.8. Polypeptides were stained by Coomassie brilliant blue. BSA (66 kD), ovalbumin (45 kD), trypsinogen (24 kD), β -lactoglobulin (18.5 kD) and lysozyme (14.3 kD) served as M_r standards. Note the similarity in polypeptide patterns of "heavy" and "light" PO (a,b) and the slight difference in M_r of the polypeptides at ~ 70 kD in c,d (arrow). Bands indicated in lane b by an asterisk (*) are contaminating polypeptides.

liver incubated with the antibodies against catalase, uricase and PMP 70. Whereas in controls, no significant differences for catalase and uricase between "heavy" and "light" POs were noted, they were both reduced in "light" POs from the regenerating livers matching the enzyme activity data (Table III). The PMP 70 exhibited a significant difference between the "light" and "heavy" preparations from both normal and partially hepatectomized animals. Whereas in "light" POs only a single band with $M_r = 70$ kD was present, in "heavy" peroxisomes consistently an additional band of $M_r = 68$ kD was visualized. This second 68-kD band was absent in the early phase after partial hepatectomy (16 h) but reappeared at 32 h (Lüers et al., 1990).

In Vivo Incorporation of Radioactive Methionine

Reliability of In Vivo Pulse Experiments. In preliminary experiments the reliability of our approach for in vivo pulse-labeling of new proteins was assessed by measuring the soluble and TCA-precipitable radioactivity (s-dpm and T-dpm) in main fractions of control rats. In Fig. 8, the TCA-precipitated ^{35}S -radioactivity (T-dpm) in the main fractions

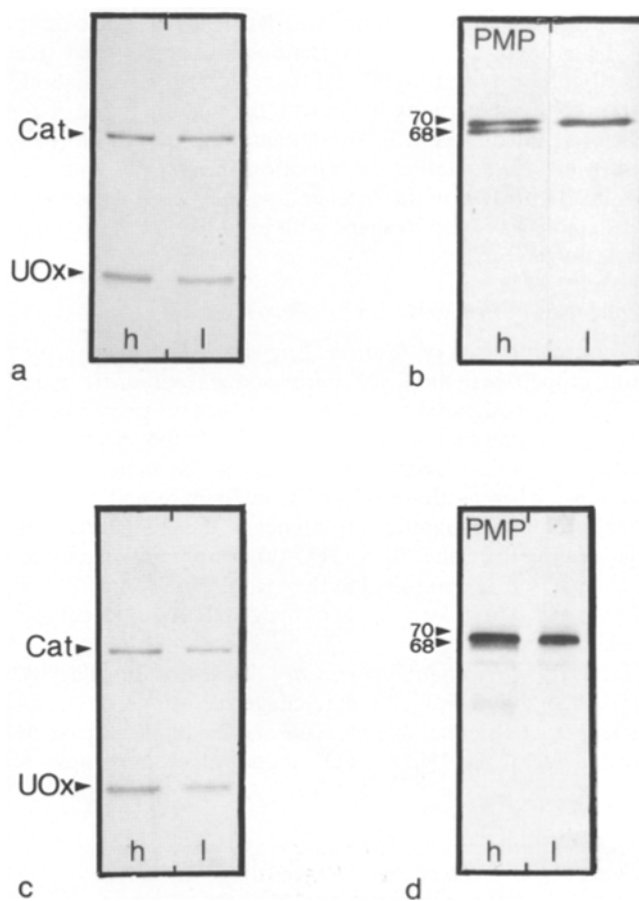


Figure 7. Immunoblots of "heavy" (h) and "light" (l) PO fractions from control (Fig. 6, a and b) and regenerating rat liver (Fig. 6, c and d), incubated for visualization of catalase (Cat), urate oxidase (UOx) and PMP 70. For Cat and UOx 0.15 μg , for PMP 70 1 μg of total peroxisomal protein was applied per lane. Further details are given in Materials and Methods. Note in the PMP-blot the presence of two bands at 70 and 68 kD in "heavy" PO in contrast to a single 70 kD band in "light" PO fractions.

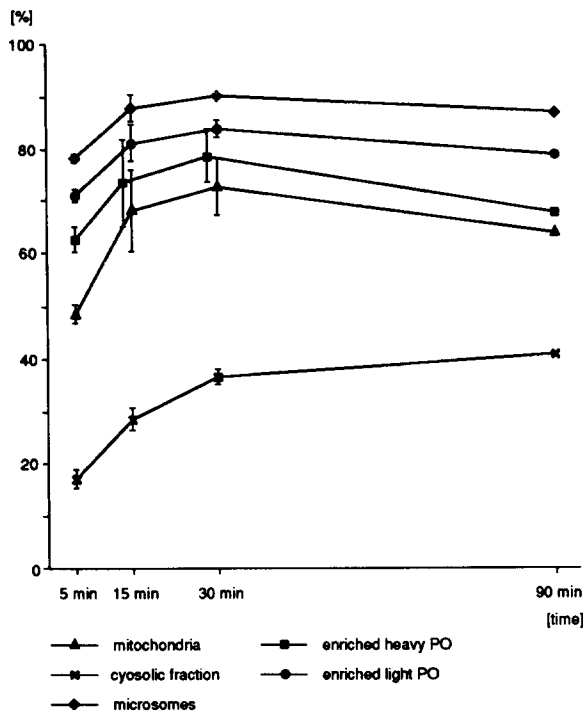


Figure 8. Rates of in vivo incorporation of [³⁵S]methionine in main subcellular fractions of control liver. TCA-precipitated ³⁵S-radioactivity in the fractions is expressed in percent of the total (TCA-precipitated and soluble) activity of those fractions. Note the uniform increase in pulse labeling of all fractions within the first 15 min. reaching a plateau thereafter. Results are mean values ± SEM of three experiments with two animals each per pulse-interval.

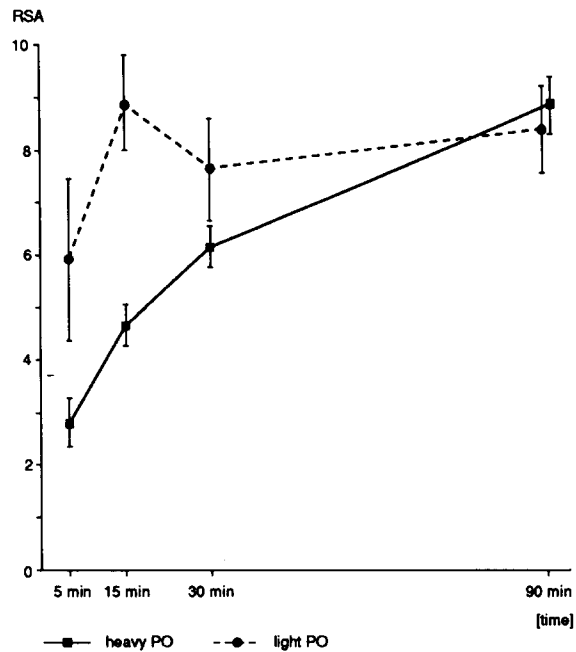


Figure 9. Kinetics of incorporation of [³⁵S]methionine into "light" and "heavy" peroxisomal fractions of control liver. Relative specific activity (RSA) × 10⁻⁴ defined as dpm/mg of peroxisomal protein over TCA-precipitable activity of total liver. Values are corrected by subtracting contributions of contaminating microsomes. Note the contrast in the initial rates of incorporation between the "light" and "heavy" PO-fractions with the two curves meeting at 90 min. Results are mean values ± SEM of at least two experiments with two animals each per pulse-interval.

expressed in percent of the total activity (T-dpm + s-dpm) of that fraction are depicted. T-dpm in all fractions attains a plateau at 15 min after the injection, remaining constant thereafter. This indicates, that the rise of label in the TCA-insoluble material of the fractions is confined to a limited length of time and that the approach matched the conditions of pulse-labeling.

Alterations of Relative Specific Activities in Control Livers. In Fig. 9, the relative rates of in vivo labeling of proteins (RSA) in "light" and "heavy" POs of control liver at 5, 15, 30, and 90 min after the injection of [³⁵S]methionine are depicted. RSA is defined as: dpm/mg of peroxisomal protein over T-dpm of total liver. Whereas RSA in "light" peroxisomes is approximately two times higher than in "heavy" POs at 5 min and reaches a maximum at 15 min, it increases progressively in the "heavy" PO fraction, matching the activity of "light" PO at 90 min.

Comparison of Peroxisomes from Control and Regenerating Livers. In regenerating liver, the rates of in vivo labeling of proteins in "light" PO also exceeded those in heavy particles two- to threefold at 5 min after the application of [³⁵S]methionine, exhibiting kinetics over the length of observation as found in control (data not shown).

Discussion

In the present study two distinct populations of PO have been

isolated from normal and regenerating rat liver by a modification of the Metrizamide-density gradient centrifugation described earlier (Völkl and Fahimi, 1985). The isolated PO differed not only morphologically in their mean diameters (Table I) and formfactors (Fig. 5) but also in their enzyme composition (Table II). Most importantly, our pulse-studies revealed, that PO-proteins are incorporated at different rates into those populations with the "light" PO showing higher initial rates of incorporation than the "heavy" PO. Whereas the specific radioactivity in the "light" PO decreased slightly during the observation period from 15–90 min, that of the "heavy" PO increased progressively suggesting a gradual transport of the radiolabel from a precursor population to the "heavy" PO.

The "Light" Peroxisomes Do Not Arise by Fragmentation From the "Heavy" Peroxisome

POs are highly fragile organelles being extremely sensitive to the homogenization and centrifugation procedures (De Duve and Baudhuin, 1966) requiring very gentle handling during their isolation (Völkl and Fahimi, 1985; Zaar et al., 1986). It could therefore be argued that the "light" PO-fractions could originate by fragmentation from the regular "heavy" PO. There are however several lines of evidence against such an argument: (a) the marked differences in the specific activities of certain enzymes in PO from control animals particularly those of the lipid-β-oxidation system (acyl-CoA oxidase and trifunctional protein) which were

significantly higher in the "light" PO, in contrast to catalase and uricase which were less divergent in the two populations; (b) the striking reduction of all enzymes only in the "light" PO after partial hepatectomy without much change of the "heavy" PO; (c) the difference in the PMP 70 between the two populations with the "light" PO containing only a single band at 70 kD and the "heavy" PO exhibiting an additional 68 kD band; (d) the marked differences in the initial rates of incorporation of radioactivity into the two subpopulations. Finally it should be noted, that the rates of recovery for catalase representing a highly soluble PO-matrix protein and urate oxidase, as an insoluble component of the peroxisomal core were essentially similar in the corresponding fractions (Table III) indicating that "light" and "heavy" PO are distinct and independent PO-subpopulations.

Ultrastructural and Enzymatic Differences Between the "Light" and "Heavy" Peroxisomes

The two peroxisome populations isolated from normal and regenerating livers differed in several respects both morphologically and biochemically. The morphometric analysis confirmed, that in normal liver the "light" PO-fraction contained significantly more POs with smaller size (mean diam: 301 nm) than the "heavy" PO fraction (mean diam: 387 nm) and although in regenerating liver those values were lower, the differences persisted (Table I). In addition, PO with irregular shapes and budding forms were more frequent in the "light" PO-fraction particularly in regenerating liver (Fig. 3). This is reflected also in the value of the formfactor which expresses the deviation of the peroxisomal shape from a circle (Table I). Those peculiar shapes and particularly the budding forms and tail-like extensions are reminiscent of in situ images reported previously in conditions associated with PO-proliferation such as in regenerating liver after partial hepatectomy (Rigatuso et al., 1970; Yamamoto and Fahimi, 1987a,b) and rat liver after treatment with hypolipidemic drugs (Baumgart et al., 1989).

Whereas in controls, the activities of two enzymes of lipid β -oxidation namely acyl-CoA oxidase and enoyl-CoA hydratase were higher in the "light" PO-fraction, the catalase activity was lower than in the "heavy" PO-fractions (Table II). The higher ratio of β -oxidation enzymes to catalase in the "light" PO-fraction is in line with observations of Flatmark et al. (1980) in rat and Klucis et al. (1991) in mouse livers after clofibrate treatment. An induction of a smaller-sized peroxisomal subpopulation in rat liver with higher levels of β -oxidation enzymes has also been observed during cold exposure (Goglia et al., 1989). Our findings in normal rat liver indicate that small PO in spite of their higher contamination by microsomes contain higher levels of β -oxidation enzymes than the "heavy" PO, implying that they are more efficient in the catabolism of very long chain fatty acids than the larger regular-sized PO. It is of interest that in regenerating rat liver the "light" PO-fraction is particularly affected in respect to the diminution of the β -oxidation enzymes (Table II). It is likely that in the course of regeneration they are not primarily engaged in lipid β -oxidation but rather provide a precursor pool for the "heavy" PO, as suggested by our pulse experiments with radioactive [35 S]methionine.

The "Heavy" and "Light" Peroxisomes Exhibit Distinct Differences in Respect to 70 kD Peroxisomal Membrane Protein

The two subpopulations of PO differed significantly in respect to the 70-kD peroxisomal membrane protein (PMP). Whereas in Western blots of the "light" PO-fractions only a single 70-kD PMP band was present, an additional immunoreactive band with $\sim M_r$ of 68 kD was consistently observed in the heavy PO-fractions. The cross-reactivity of the antibody to 70 kD PMP with another 68-kD protein in the isolated peroxisomal membrane fractions was also described in earlier reports (Hashimoto et al., 1986; Baumgart et al., 1989) and it has been suggested, that it is a product of the proteolytic cleavage of the 70-kD PMP because its formation can be suppressed by the addition of leupeptin to the isolation medium (Hashimoto et al., 1986). The susceptibility of the large PMP to proteolytic cleavage was also described by Hartl and Just (1987), although the formation of the 68-kD product was not mentioned. A 68- and 70-kD PMP has also been described in PO of the mouse liver, yet at variance with rat hepatic PO, those proteins were considered to be structurally unrelated (Chen et al., 1988). The exclusive appearance of the 68-kD band in the "heavy" PO-fraction implies that the 70-kD PMP in that fraction is more susceptible to proteolysis than in the "light" PO-fraction. This would suggest a "maturation" of the 70-kD PMP during the transition from the "light" to the "heavy" PO. This notion is also supported by the finding that the 68-kD band is absent in "heavy" PO of the early phase (16 h) after partial hepatectomy, with its beginning formation of new PO, but reappears at the later stage (32 h) of regeneration when mature PO gradually reappear (Lüers et al., 1990). This interpretation deserves special interest in respect to the role of the 70-kD PMP as a putative ATP-binding protein involved in ATP-driven transport of small molecules or proteins through membranes (Kamijo et al., 1990). Irrespective of those interpretations, the 68-kD PMP seems to serve as a useful marker for the identification of "heavy" PO-fractions in contradistinction to the "light" ones and should be helpful in further characterization of those subpopulations.

The "Light" and "Heavy" Peroxisomes Exhibit Distinct Rates of In Vivo Pulse-labeling

The in vivo pulse labeling system used in the present study was originally introduced by Lazarow and de Duve (1971) and its reliability is documented in Fig. 8, which shows the rapid rise of label in the main fractions reaching a plateau within ~ 15 min and remaining stable during the rest of the observation period (up to 90 min). The direct injection of [35 S]methionine into the portal vein most probably facilitated its instantaneous and uniform access to hepatocytes, thus fulfilling a major prerequisite for true in vivo pulse-labeling (Peters et al., 1962; Lazarow and de Duve, 1971).

The quantity and kinetics of incorporation of radioactivity differed markedly in the two peroxisomal fractions with the "light" PO containing two fold higher label initially than the "heavy" PO fractions (Fig. 9). This suggests strongly that the new proteins are preferentially incorporated into the "light" PO-fractions. Moreover, during the 90-min observation period the RSA in "light" PO decreased insignificantly

while it progressively increased in the "heavy" PO-fractions. It must be emphasized, however, that the rate of diminution of radioactivity in the "light" PO did not match its elevation in the "heavy" ones. This could be either due to a partial contamination of the "light" PO by some "heavy" ones or alternatively due to the existence of still smaller POs which would incorporate even more efficiently the radioactivity passing it on to the "light" PO. Irrespective of the exact composition of the "light" PO, the evident gradual increase of radioactivity in the "heavy" PO over the 90 min observation period strongly suggests that they serve as recipients of the newly synthesized proteins from a precursor population.

Recently, Heinemann and Just (1992) studied in vitro the biosynthesis of acyl-CoA oxidase by pulse-chase labeling in isolated hepatocytes from rats treated with clofibrate, and also noted that the newly synthesized protein was first (at 7.5 min) imported into a peroxisomal fraction with a lower density (1.16 g/cm³) before reaching at 30 min the mature POs with a density of 1.23 g/cm³. Both their report and our observations here are at variance from the classical study of Poole et al. (1970). Those authors however, failed to study the early period (up to 90 min) of incorporation of radioactivity into proteins which seems to be crucial for the biosynthesis of PO proteins. Indeed, the earliest interval they investigated was 180 min. Our observations however show that although in the initial 15 min. the RSA in "light" and "heavy" POs diverge by 100%, they reach equilibrium at 90 min, as reported by Poole et al. (1970).

In summary, two subpopulations of PO, "light" and "heavy" have been isolated from rat liver, which differ both by ultrastructural and biochemical criteria. Most interestingly, they also differ in the rate of incorporation of newly synthesized peroxisomal proteins with kinetic data suggesting that "light" PO may serve as precursors to the regular "heavy" PO. The exact mode of transport of newly synthesized proteins from the "light" to "heavy" POs is not known and should be a rewarding task for future investigations.

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