Water- and solute-accessible spaces of purified peroxisomes

Evidence that peroxisomes are permeable to NAD⁺

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Peroxisomes were purified from liver homogenates from rats, treated with the peroxisome proliferator clofibrate, by a combination of differential centrifugation and isopycnic centrifugation in iso-osmotic self-generating Percoll gradients. Structural integrity of the peroxisomes appeared to be preserved as evidenced by a high degree of catalase latency, the absence of catalase release during purification and the exclusion of inulin (mol.wt. $\pm$ 5000). Spaces for water and solutes were measured after incubation of the peroxisomes in iso-osmotic sucrose with radioactive water or solutes and separation of the organelles from their media by centrifugation through an organic layer. Extraperoxisomal water was corrected for by the use of radioactive dextran or inulin. The sucrose, glucose, urea, methanol and acetate-accessible spaces were identical, suggesting that these spaces represent the volume in which molecules that can cross the membrane distribute. This volume equalled 50–65% of the water space. Urate and NAD⁺, a cofactor of peroxisomal β -oxidation of fatty acids, also distributed in this volume, but were also partly bound. Urate and NAD⁺ binding was not abolished by sonication, which released the bulk of matrix catalase activity, but NAD⁺ binding was seriously diminished. The peroxisomal water and sucrose spaces were estimated to be 107 μ l and 55 μ l per g of liver tissue from a clofibrate-treated rat. From quantitative morphometric data [Anthony, Schmucker, Mooney & Jones (1978) *J. Lipid Res.* **19**, 154–165] and our marker enzyme analyses, as well as from our experimentally determined water spaces of mitochondrial and microsomal fractions, it could be calculated that the volume contamination by lysosomes, mitochondria and microsomes did not exceed 1, 8 and 6% respectively. Our data indicate that apparently intact peroxisomes are permeable to a number of small molecules, including NAD⁺. Whether the NAD⁺-binding sites in sonicated peroxisomes mirror the likely existence of a membrane carrier requires further investigation.

The discovery of a fatty acid-oxidizing system in glyoxysomes from castor-bean endosperm (Cooper & Beevers, 1969) and later in peroxisomes from rat liver (Lazarow & de Duve, 1976) has raised the question as to the permeability of the peroxisomal membrane to cofactors such as nicotinamide nucleotides, CoA and carnitine. From the centrifugal behaviour of peroxisomes in sucrose density gradients and the lack of latency of peroxisomal oxidases, it has been concluded that peroxisomes are permeable to sucrose and to the substrates of their oxidases (de Duve & Baudhuin, 1966; Baudhuin, 1969). In the sucrose density gradients commonly used for their purification, peroxisomes equilibrate at a density of about 1.24 g $\cdot$ cm⁻³ (approx. 1.9 M-

sucrose) (Tolbert & Essner, 1981). The ensuing uncertainties with regard to structural damage and permeability changes caused by the dense sucrose solution or by its dilution to iso-osmolarity have hitherto precluded direct permeability measurements on purified peroxisomes.

In the present paper we describe the direct determination of the spaces for water, sucrose and other solutes of peroxisomal fractions purified by isopycnic centrifugation in self-generating iso-osmotic Percoll gradients. The peroxisomes were therefore kept under iso-osmotic conditions throughout the entire purification procedure. The spaces were measured after incubation of the purified peroxisomes with radioactive water or solutes and

separation of the organelles from their incubation medium by centrifugation through an organic layer. In a first series of experiments various small molecules known or suspected to cross the peroxisomal membrane were studied. Once the space accessible to these molecules was established, it could be used as a measure for comparison with spaces obtained for cofactors of fatty acid oxidation. The present paper reports the permeability of peroxisomes to NAD^+ . Amongst the cofactors of fatty acid oxidation, NAD^+ was studied first because evidence from our laboratory suggested that it might penetrate the peroxisome: addition of NAD^+ is an absolute requirement for acyl-CoA oxidation assayed in either disrupted or intact organelles (Mannaerts *et al.*, 1979; Thomas *et al.*, 1980) and purified peroxisomes do not contain NAD^+ or NADH (Mannaerts *et al.*, 1982). This is in sharp contrast with CoA, which is present in purified peroxisomes (Van Broekhoven *et al.*, 1981), the addition of which does not stimulate acyl-CoA oxidation in intact peroxisomes.

Experimental

Animals

Male Wistar rats weighing 200g were fed a regular chow diet containing 0.3% clofibrate for 2 weeks. Clofibrate, a hypolipidaemic agent, causes the proliferation of peroxisomes in rodent liver (Hess *et al.*, 1965).

Cell fractionation and purification of peroxisomes

Liver homogenates (25%, w/v) prepared in 0.25 M-sucrose/0.1% (v/v) ethanol/5 mM-dithiothreitol (homogenization medium) were fractionated by differential centrifugation and the λ -fractions, enriched in lysosomes and peroxisomes, were subfractionated by centrifugation in self-generating 30% iso-osmotic Percoll gradients (Mannaerts *et al.*, 1982). The gradient fractions most enriched in peroxisomes were pooled, diluted 5-fold with homogenization medium and the peroxisomes sedimented under the same conditions as the original λ -fractions, resulting in a loosely (because of the presence of Percoll particles) packed pellet of purified peroxisomes (Mannaerts *et al.*, 1982). To remove the remaining Percoll particles, which caused high blank values in the permeability assays, the purified peroxisomes were resuspended in homogenization medium, brought to 10 $\times$ the volume of the pooled gradient fractions and again sedimented as described above, resulting this time in a firm pellet. This final pellet was then resuspended in homogenization medium to a protein concentration of 1–1.5 mg/ml and used in the permeability measurements.

Enzyme assays

Marker enzyme (catalase and urate oxidase for peroxisomes, glutamate dehydrogenase for mito-

chondria, glucose 6-phosphatase for microsomes and acid phosphatase for lysosomes) activities were determined as described previously (Mannaerts *et al.*, 1982). Esterase activity (microsomes) was measured as described by Beaufay *et al.* (1974). Protein was determined by the method of Peterson (1977).

Catalase, a marker enzyme for the peroxisomal matrix, shows latency. Therefore free (non-latent) and total catalase activities were measured (Mannaerts *et al.*, 1982). Free catalase activity, expressed as the percentage of total catalase present in a particular preparation, was taken as an index for the structural integrity of the peroxisomes present in that preparation. Approx. 40–50% of the catalase activity present in a liver homogenate is non-latent and cannot be sedimented by centrifugation. It still is an open question whether this catalase is truly soluble in the intact cell or whether it is released during homogenization. Electron micrographs of peroxisomes present in λ -fractions show significant matrix extraction (Leighton *et al.*, 1975). Nevertheless, the organelles are surrounded by a continuous and apparently intact membrane and catalase present in these fractions shows a high degree of latency. These observations suggest that during homogenization the soluble catalase activity may originate from the partial release of catalase from peroxisomes that subsequently restore their structural integrity and permeability rather than from the complete disruption of part of the organelles. It is therefore appropriate to use the sedimentable catalase activity of liver homogenates instead of their total catalase in calculations of the peroxisomal yield of purified preparations (Table 1).

Determination of peroxisomal water and solute spaces: permeability assays

For the determination of the peroxisomal spaces for water and solutes purified peroxisomes were incubated with radioactive water or solutes and separated from their incubation medium by centrifugation through an organic layer of brominated hydrocarbons (Cornell, 1980). Eppendorf tubes (1.5 ml) contained a bottom layer (0.05 ml) of homogenization medium made 0.396 M in sucrose ($d = 1.051$), a middle layer (0.4 ml) consisting of a mixture of bromododecane and bromodecane (8:2, v/v; $d = 1.044$) and an upper layer (0.55 ml), which, after the addition of purified peroxisomes, consisted of 0.24 M-sucrose, 20 mM-Tricine {*N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine}, pH 7.2, 5 mM-dithiothreitol and 0.1% (v/v) ethanol. The upper layer also contained $^3\text{H}_2\text{O}$ or the radioactive solutes at a concentration of 0.5 mM, except for sucrose (0.24 M), methanol (31 mM) and dextran and inulin, which were used without dilution of the radioactivity. In all cases radioactivity amounted to

approx. $1.8 \mu\text{Ci/ml}$. Incubations were started by the addition of 0.2–0.3 mg of peroxisomal protein. After 5 min of incubation at room temperature the tubes were centrifuged in an Eppendorf table centrifuge 5414 at 9980 g for 7 min and the upper layer and organic middle layer were aspirated. The bottom layer containing the sedimented peroxisomes was mixed with 0.1 ml of 0.2% (v/v) Triton X-100 and a portion was solubilized with Soluene and counted for radioactivity. Spaces were expressed in μl per unit of sedimented catalase. When enzyme activities were measured in the bottom layer, incubations and centrifugations were performed in the absence of radioactivity. Recoveries of enzyme activities and of protein in the upper and bottom layers after centrifugation reached approx. 70% (compare data of Tables 1 and 3). The reason is that some material that could not be accurately recovered accumulated at the water/hydrocarbon interfaces. All measurements of intraperoxisomal spaces were corrected for adhering extraperoxisomal water by subtracting the dextran or inulin spaces, which were equal (see the legend to Table 2). Appropriate blanks in the absence of peroxisomal protein were also performed. The incubation time of 5 min amply sufficed for equilibration of radioactivity between the incubation media and the peroxisomal compartments, since equilibrium was already reached when centrifugation was started without prior incubation. The measured peroxisomal spaces for water and solutes increased linearly with increasing amounts of added peroxisomal protein or catalase. In some experiments the purified peroxisomes were disrupted by sonication (Branson Sonifier B-15P Cell Disrupter, output 5, 50% duty cycle) for 20 s in ice, resulting in the release of more than 80% of the catalase. The disrupted organelles were incubated and centrifuged as described for the intact organelles.

Fractions enriched in mitochondria or microsomes were prepared by differential centrifugation (Debeer *et al.*, 1979) and used in the permeability assays as described for purified peroxisomes.

Calculation of the volume of contaminating organelles

The volume of mitochondria, microsomes and lysosomes contaminating the purified peroxisomal preparation was estimated by two approaches.

A first approach was based on the quantitative morphometric data obtained by Anthony *et al.* (1978) and by Van Hoof *et al.* (1979) for livers of male clofibrate-treated rats. These authors estimated the volume fraction (fraction of volume of the hepatocyte occupied by the various structures) to be 0.29 (Van Hoof *et al.*, 1979) or 0.25 (Anthony *et al.*, 1978) for mitochondria, 0.063 (Van Hoof *et al.*, 1979) or 0.076 (Anthony *et al.*, 1978) for peroxi-

somes and 0.0067 (Anthony *et al.*, 1978) for lysosomes. The total surface of rough and smooth endoplasmic reticulum was estimated to be $13.82 \text{ m}^2/\text{ml}$ of liver tissue or $15.53 \text{ m}^2/\text{ml}$ of hepatocyte (Anthony *et al.*, 1978). The number of spherical microsomal vesicles with radius R that can originate from an endoplasmic reticulum surface S equals $S/(4\pi R^2)$ and their total volume equals $(4\pi R^3)/3 \times S/(4\pi R^2)$ or $(R \times S)/3$. Depending on whether the average radius of the microsomal vesicles is taken as 0.05 or $0.1 \mu\text{m}$, their volume would equal 0.26 or 0.52 ml per ml of hepatocyte, which gives volume fractions 0.26 and 0.52. Knowing the volume fractions (relative volumes) of the various organelles and knowing the percentage recovery of the various organelles in the sediment after centrifugation of the purified peroxisomal preparation through the organic layer (Table 1), one can calculate the relative volumes of the contaminating organelles present in the peroxisomal sediment.

In a second approach, the apparent water spaces of fractions enriched in mitochondria and microsomes were determined as described in the preceding paragraph and the data expressed per unit of glutamate dehydrogenase (mitochondria) or per unit of esterase (microsomes). By using these values and the activities of glutamate dehydrogenase and esterase recovered in the bottom layer after centrifugation of the purified peroxisomes through the hydrocarbon layer, the contribution of contaminating mitochondria and microsomes to the overall water space of the peroxisomal preparation could be calculated.

Materials

Bromododecane and bromodecane were purchased from Aldrich Europe. Percoll was from Pharmacia and Soluene-350 from Packard. [$1\text{-}^{14}\text{C}$]Acetic acid (sp. radioactivity 59.5 Ci/mol), [3H]dextran (sp. radioactivity 0.14 Ci/g), [^{14}C]methanol (sp. radioactivity 58 Ci/mol), [^{14}C]urea (sp. radioactivity 54.4 Ci/mol), [$1\text{-}^{14}\text{C}$]uric acid (sp. radioactivity 60 Ci/mol), [$4\text{-}^3\text{H}$]NAD (sp. radioactivity 2.29 Ci/mol) and $^3\text{H}_2\text{O}$ (sp. radioactivity 0.005 Ci/ml) were from The Radiochemical Centre, Amersham, Bucks., U.K. [$\text{U-}^{14}\text{C}$]Glucose (sp. radioactivity 2.4 Ci/mol), [$\text{G-}^3\text{H}$]inulin (sp. radioactivity 0.14 Ci/g) and [$\text{U-}^{14}\text{C}$]sucrose (sp. radioactivity 602 Ci/mol) were from New England Nuclear.

Results and discussion

Characteristics of the purified peroxisomal preparation

Absolute activities of marker enzymes in whole homogenates and recoveries at different stages of

Table 1. *Composition of the purified peroxisomal pellet and the sediment obtained after centrifugation through an organic layer of brominated hydrocarbons*

Results are expressed as percentages of total homogenate activity and relative specific activities. Relative specific activity (de Duve *et al.*, 1955) is defined as the percentage of total activity present in a particular preparation divided by the corresponding percentage of total protein. Results are means $\pm$ S.E.M. for the numbers of experiments indicated in parentheses.

	Purified peroxisomal pellet		Sediment	
	% of total homogenate	Relative specific activity	(% of total homogenate)	Relative specific activity
Protein (15)	0.49 $\pm$ 0.04	—	0.34 $\pm$ 0.03	—
Catalase (14)	7.96 $\pm$ 0.45	16.24	4.55 $\pm$ 0.47	13.38
Sedimentable catalase (12)	12.03 $\pm$ 0.92	24.55	7.16 $\pm$ 1.04	21.06
Glutamate dehydrogenase (4)	0.34 $\pm$ 0.10	0.69	0.22 $\pm$ 0.09	0.65
Glucose 6-phosphatase (3)	0.23 $\pm$ 0.04	0.47	0.15 $\pm$ 0.02	0.44
Esterase (3)	0.14 $\pm$ 0.04	0.29	0.09 $\pm$ 0.04	0.26
Acid phosphatase (4)	0.94 $\pm$ 0.03	1.92	0.90 $\pm$ 0.07	2.65

purification have been published previously (Mannaerts *et al.*, 1982). Table 1 shows the composition of the final purified peroxisomal pellet and of the sediment obtained after centrifugation of the purified peroxisomes through the organic layer during the permeability assays. Organelle purification is expressed as relative specific activities of marker enzymes, which indicate the purification factor from the homogenate on a protein basis. It should be noted, however, that because of the severalfold increase in peroxisomal protein after clofibrate treatment, the relative specific activities that can be obtained in purified peroxisomal preparations from clofibrate-treated rats are never as high as those found in preparations from untreated rats. The Table further shows that 60–70% of the peroxisomes and contaminating microsomes and mitochondria were recovered in the bottom layer after centrifugation through the organic layer, whereas almost all contaminating lysosomes sedimented.

The structural integrity of the peroxisomes appeared to be well preserved during the purification. Free catalase activity of the original λ -fractions loaded on the Percoll gradients was 11% as compared with 15% for the final peroxisomal preparation. Since free catalase activity of a granule fraction never decreases below 10–15% (de Duve & Baudhuin, 1966) this percentage seems compatible with a perfectly intact membrane. Even after centrifugation through the organic layer, free catalase activity amounted to only 20% (means for eight experiments). Peroxisomes can be damaged by the hydrostatic pressure generated during centrifugation (Bronfman & Beaufay, 1973; Wattiaux *et al.*, 1971). Whereas catalase is easily released from the damaged peroxisomes, urate oxidase is not. Therefore urate oxidase was also measured in some experiments. The ratio of the percentage sedimentable catalase over the percentage urate oxidase

present in the peroxisomal preparations did not decline throughout the purification procedure, being 1.10, 1.28 and 1.20 in λ -fractions, purified peroxisomes and peroxisomes sedimented through the organic layer respectively (means for three experiments). This indicates no loss of catalase relative to urate oxidase. Finally, since dextran (mol.wt. 79 000) and inulin (mol.wt. 5000) spaces were equal (see the legend to Table 2), the peroxisomes were not permeable to compounds with a mol.wt. $\geq$ 5000.

Peroxisomal spaces for water and solutes

In a first series of experiments we wanted to study the permeability of peroxisomes to small molecules that from indirect evidence were thought to enter the peroxisomes. The purpose was to determine an intraperoxisomal solute-accessible space that could serve as a basis for comparison with spaces obtained for cofactors of peroxisomal β -oxidation such as NAD⁺. From their behaviour in sucrose-density gradients it has been concluded that peroxisomes are permeable to sucrose (de Duve & Baudhuin, 1966; Baudhuin, 1969). However, Wattiaux and co-workers (see Wattiaux, 1973) have shown that the hydrostatic pressure generated during high-speed centrifugation in dense sucrose solutions renders membranes such as the mitochondrial inner membrane permeable to sucrose, casting some doubt on the peroxisomal permeability for sucrose under physiological circumstances. We determined the sucrose permeability of peroxisomes that had been isolated under iso-osmotic conditions and that had been subject to much less hydrostatic pressure because of the use of relatively low centrifugal forces and an angular rotor instead of a swing-out rotor. The peroxisomal space measured for sucrose was then compared with spaces obtained for molecules known to be metabolized by intact peroxisomes

Table 2. *Peroxisomal spaces for water and various solutes*

Peroxisomal spaces were measured after incubation of purified peroxisomes with radioactive water or solutes and separation of the organelles from their incubation media by centrifugation through an organic layer of brominated hydrocarbons. Measurements are corrected for extraperoxisomal spaces determined with radioactive dextran or inulin. Results are expressed as μl per unit of catalase and are means $\pm$ S.E.M. for the numbers of experiments indicated in parentheses. Catalase units are defined as described by Baudhuin *et al.* (1964). Extraperoxisomal space was $2.35 \pm 0.21 \mu\text{l}/\text{unit}$ of catalase ($n = 10$) and $2.36 \pm 0.26 \mu\text{l}/\text{unit}$ of catalase ($n = 4$) as determined with dextran and inulin respectively.

Space	Peroxisomal space ($\mu\text{l}/\text{unit}$ of catalase)	Solute-accessible space/water space
Water (9)	$2.52 \pm 0.21 \ddagger$	—
Sucrose (6)	$1.30 \pm 0.08 \dagger$	0.52
Glucose (3)	1.63 ± 0.30	0.65
Methanol (4)	$1.37 \pm 0.28 \dagger$	0.54
Urea (5)	$1.56 \pm 0.14 \dagger$	0.62
Acetic acid (5)	$1.25 \pm 0.25 \dagger$	0.50
Uric acid (7)	$2.12 \pm 0.23 \ddagger$	0.84
NAD ⁺ (5)	$3.20 \pm 0.37 \ddagger$	1.27

$\dagger$ Significantly ($P < \text{or} \leq 0.05$) different from the water space as determined by Dunnett's adaptation for one control and several treatments of Student's *t* test (Li, 1964).

$\ddagger$ Significantly ($P < \text{or} \leq 0.05$) different from the sucrose space.

(urate and methanol) or molecules known to diffuse easily through some other biological membranes.

Table 2 shows the peroxisomal space for water and the various small solutes. All measurements were corrected for extraperoxisomal adhering water. The sucrose, methanol, urea and acetate-accessible spaces differed significantly from the water space but not from each other. The glucose-accessible space was significantly different from the water space when differences were tested within the same experiments with Student's paired *t* test [glucose: $1.63 \pm 0.30 \mu\text{l}/\text{unit}$ of catalase; water: $3.20 \pm 0.25 \mu\text{l}/\text{unit}$ of catalase ($n = 3$; difference significant at $P < 0.01$)]. No difference was observed, however, with the sucrose space. These results indicate that peroxisomes are permeable to various uncharged and charged small molecules and suggest that the sucrose-accessible space represents the volume in which molecules that are able to cross the peroxisomal membrane distribute. This space equals only 50–65% of the peroxisomal water space. Part of this difference may be accounted for by hydration water and by contamination of the peroxisomal preparation with organelles, such as

microsomes and mitochondria, that may not be accessible for the solutes. The latter possibility is unlikely, however, since microsomes are permeable to sucrose, glucose (Nilsson *et al.*, 1973; Meissner & Allen, 1981) and, most likely, urea and mitochondria are permeable to urea and acetate. In addition, contamination appears to be small (see below). It seems therefore that a small part of the intraperoxisomal space may not be easily penetrable for the tested compounds.

The urate-accessible space was significantly larger than the sucrose space. This could indicate that urate, which, from the lack of latency of urate oxidase, is known to cross the peroxisomal membrane, might distribute into a larger volume than sucrose and the other solutes or that urate might bind to peroxisomes. To discriminate between these possibilities, spaces were determined over a range of solute concentrations. The amount of radioactivity was kept constant. As can be seen from Fig. 1(a) the urea- and acetate-accessible spaces were concentration-independent, as is to be expected for compounds taken up by diffusion. In the case of urate, however, the apparent space greatly exceeded the sucrose space at low urate concentrations, but sharply declined to reach values only slightly larger than the sucrose space at high concentrations, suggesting the occurrence of a rapidly saturating binding of rather low capacity. Supporting evidence for this is presented in Fig. 1(b) in which urate uptake is plotted against urate concentration. The shape of the curve suggests that it is composed of two components: a saturable one (binding) and a non-saturable one that increases linearly with increasing urate concentrations (diffusion). When the curve is corrected for urate uptake by diffusion into the sucrose space, the saturable component becomes apparent. This was also the case when urate uptake was measured in peroxisomes that had been disrupted by sonication (Fig. 1c). In these experiments the dextran- and sucrose-accessible spaces were almost equal (see Table 3) so that after correction for the dextran space almost no net sucrose space was left, indicating nearly complete disruption of the peroxisomes. When urate uptake was corrected for diffusion in the dextran space a saturation curve was obtained that closely resembled the curve obtained in intact peroxisomes after correction for diffusion in the sucrose space. These experiments therefore indicate that the intraperoxisomal distribution volumes for urate, sucrose and the other tested compounds are identical. In addition, there appears to be a finite number of binding sites for urate. The nature of these binding sites, which are not located in the peroxisomal matrix since catalase was almost completely released by sonication (see Table 3), remains to be determined. A likely possibility is that part of the urate and its conversion product allantoin

Fig. 1. Urate uptake by purified peroxisomes

Purified peroxisomes were incubated with radioactive solutes and solute-accessible spaces (a) or solute uptake (b and c) were determined after centrifugation of the peroxisomes through an organic layer. (a) ●—●, apparent urate-accessible space (mean for two experiments); ■—■, acetate-accessible space; ▲—▲, urea-accessible space. Urate, acetate and urea spaces were determined on separate batches of purified peroxisomes. (b) ●, Total peroxisomal urate uptake; ○, total urate uptake minus calculated urate uptake by diffusion into the sucrose-accessible space. Same experiments as in (a). (c) □, Total urate uptake by peroxisomes disrupted by sonication. Since catalase was largely released after sonication, results are not expressed per unit of catalase but per mg of protein. Same experiments as in (a) and (b). For reference, urate uptake by intact peroxisomes, corrected for diffusion into the sucrose space (see b), is also expressed per mg of protein (○).

Table 3. *Characteristics of sonicated peroxisomes*
 Peroxisomes were sonicated and centrifuged through the brominated hydrocarbon layer as described in the Experimental section. Since catalase was largely released by sonication, spaces are expressed per mg of protein and are not corrected for extraperoxisomal (= dextran) space. Enzyme release is expressed as the percentage of enzyme activity that did not sediment through the organic layer and that was recovered in the top layer after centrifugation. The experiments described in this Table include the experiments depicted in Figs. 1 and 2. Values are means $\pm$ S.E.M. for the numbers of experiments indicated in parentheses.

	Untreated peroxisomes	Sonicated peroxisomes
Space (μl/mg of protein)		
Water	17.09 $\pm$ 1.67 (10)	13.93 $\pm$ 1.58 (8)
Sucrose	12.33 $\pm$ 1.09 (10)	13.33 $\pm$ 1.63 (8)
Dextran	7.97 $\pm$ 0.87 (10)	11.16 $\pm$ 1.61 (8)
Enzyme release (%)		
Catalase	8.2 $\pm$ 1.0 (5)	85.5 $\pm$ 5.5 (8)
Urate oxidase	3.0 (2)	26.8 (2)
Protein	4.9 $\pm$ 1.3 (5)	30.0 $\pm$ 2.3 (8)

are bound by urate oxidase itself, even after sonication, since the bulk of urate oxidase could not be recovered in the top layer after centrifugation (Table 3). A non-equilibrium between continuously generated intraperoxisomal allantoin and extraperoxisomal allantoin might also mimic binding. Finally, the occurrence of urate-binding sites at the peroxisomal membranes, possibly reflecting a membrane carrier, cannot be excluded either.

Peroxisomal permeability to NAD^+

When used at a concentration of 0.5 mM, the apparent peroxisomal space for NAD^+ was considerably larger than that for sucrose and even water, suggesting that like urate, NAD^+ might bind to peroxisomes. Therefore the same kind of experiments was performed with NAD^+ as with urate (see Fig. 2). As in the case of urate, the apparent space accessible to NAD^+ sharply declined with increasing NAD^+ concentration, to reach values that closely approached the size of the sucrose space (Fig. 2a). Fig. 2(b) shows that when NAD^+ uptake was plotted against NAD^+ concentration the resulting curve was composed of two components: a saturable one (binding) that becomes apparent when NAD^+ uptake is corrected for diffusion into the sucrose space, and a non-saturable one (diffusion) that increases linearly with the NAD^+ concentration. Our results therefore indicate that NAD^+ penetrates the peroxisomes and that its distribution volume equals that of sucrose and the other tested solutes.

Fig. 2. NAD^+ uptake by purified peroxisomes. Purified peroxisomes were incubated with radioactive NAD^+ and the NAD^+ -accessible space (a) or NAD^+ uptake (b and c) were determined after centrifugation of the peroxisomes through an organic layer. (a) $\bullet$, Apparent NAD^+ -accessible space (mean for three experiments). (b), $\bullet$, Total peroxisomal NAD^+ uptake; $\circ$, total NAD^+ uptake minus calculated NAD^+ uptake by diffusion into the sucrose-accessible space. Same experiments as in (a). (c), $\square$, Total NAD^+ uptake by peroxisomes disrupted by sonication. Since catalase was largely released after sonication, results are not expressed per unit of catalase but per mg of protein. Results are means for four experiments, which are different from the experiments represented in (a) and (b). For reference, NAD^+ uptake by intact peroxisomes, corrected for diffusion into the sucrose space (see b) is also expressed per mg of protein ($\circ$).

No formation of NADH could be detected during the permeability assays, likely because of the lack of fatty acyl-CoA substrate for β -oxidation. NAD^+ binding was not abolished by sonication but seriously diminished. This could mean that NAD^+ is mainly bound by matrix components, probably β -hydroxyacyl-CoA dehydrogenase. The binding to sonicated peroxisomes might indicate that, unlike catalase, some matrix components remained associated with the peroxisomal membranes. However, the intriguing possibility remains that it might be the expression of binding sites associated with the membrane itself, which in turn might reflect the likely occurrence of a membrane carrier for NAD^+ . Peroxisomal membranes, stripped of their peripheral proteins, which were prepared by carbonate treatment of purified peroxisomes (Fujiki *et al.*, 1982), displayed only negligible NAD^+ binding. However, NAD^+ binding to sonicated peroxisomes was strongly inhibited by NADH, Cibacron Blue and mersalyl. When NAD^+ was incubated for time periods of less than 1 min with intact peroxisomes in the presence of these agents, the NAD^+ -accessible space became smaller than the sucrose-accessible space (P. Van Veldhoven, unpublished work), indicating that these agents do not only interfere with NAD^+ binding but also with NAD^+ diffusion in the peroxisome.

Volume and protein content of peroxisomes in liver tissue

By quantitative morphometric analysis Anthony *et al.* (1978) have estimated the peroxisomal volume at 11 and 68 μ l per ml of liver tissue for livers from control and clofibrate-treated rats respectively. The corresponding values given by Van Hoof *et al.* (1979), after correction for hepatocyte volume per ml of liver tissue, would be 9 and 56 μ l per ml of liver tissue. Since the liver homogenates in our experiments contained 42.3 ± 1.4 ($n = 16$) units of sedimentable catalase per g of tissue and since there was no further loss of catalase from the peroxisomes during the purification procedure (see above), use of this conversion factor allows the calculation of the peroxisomal volume per g of tissue. The total peroxisomal water space per g of liver tissue from a clofibrate-treated rat would equal 107 μ l and the sucrose-accessible peroxisomal space 55 μ l.

Our purified peroxisomes contained 0.18 mg of protein per unit of catalase. From the sedimentable catalase activity it can be calculated that peroxisomes would account for approx. 8 mg of protein per g of liver from a clofibrate-treated rat. Assuming that all soluble catalase activity (approx. 40%) is released from peroxisomes injured during the homogenization, total peroxisomal protein could amount to a maximum of 13 mg of protein per g. This value,

which is somewhat overestimated owing to the presence of contaminating organelles, equals half the estimation given by Lazarow & de Duve (1976) for livers from clofibrate-treated animals and is only twice the value of 6.5 mg per g of liver from a normal rat as estimated by Leighton *et al.* (1968). Clofibrate treatment of rats results in a 5–10-fold increase in the number of hepatic peroxisomes.

Volume of contaminating organelles

Using quantitative morphometric data (Anthony *et al.*, 1978; Van Hoof *et al.*, 1979) and the marker enzyme activities recovered after centrifugation of the purified peroxisomes through the organic layer (Table 1), one can calculate that the volume contamination by lysosomes would be approx. 1%, by mitochondria 8% and by microsomes 4–8%, depending on whether the average radius of microsomal vesicles is taken as 0.05 or 0.1 μm . We therefore determined the water space of fractions enriched in mitochondria or microsomes that were prepared by differential centrifugation. The water spaces of the mitochondrial and microsomal fractions were $1.44 \pm 0.18 \mu\text{l}$ per unit of glutamate dehydrogenase ($n = 6$) and $1.21 \pm 0.23 \mu\text{l}$ per unit of esterase ($n = 4$) respectively. From the recovery of these marker enzymes in the bottom layers after centrifugation of the peroxisomal preparations through the brominated hydrocarbons it could be calculated that the water volume contamination by mitochondria was 9.2% and by microsomes 6.2%. The volume contamination by mitochondria is an overestimation, however, since mitochondrial fractions prepared by differential centrifugation are seriously contaminated by other organelles such as peroxisomes and lysosomes. Any marked contamination by microsomes was further ruled out by the fact that the acetate-accessible space was not significantly smaller than the spaces for sucrose, glucose or urea (Table 2). Acetate does not penetrate the microsomal vesicles (Nilsson *et al.*, 1973), a finding that was confirmed by our experiments (results not shown).

Conclusion

We have performed direct permeability measurements on peroxisomes that were kept under isotonic conditions during the whole purification procedure and that had preserved their structural integrity as judged from measurements of catalase latency and from the exclusion of inulin and dextran. Strictly, our data only apply to proliferated peroxisomes, but it is unlikely that 'normal' peroxisomes would display different permeability characteristics. Evidently, as applicable to all isolated organelles, discrete permeability changes caused by the isolation procedure cannot be excluded. The peroxisomal

preparation and permeability assay described in this communication should allow the determination of the permeability of peroxisomes to the various cofactors for β -oxidation as has been illustrated here for NAD^+ . The permeability of peroxisomes for NAD^+ , and most likely for NADH, suggests that NADH produced during β -oxidation of fatty acids does not need to be re-oxidized within the peroxisome but that the peroxisomal redox state could be regulated by the redox state of the cytosolic nicotinamide nucleotides.

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