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*Published in:*  
Archives of Microbiology

*DOI:*  
[10.1007/BF00248432](https://doi.org/10.1007/BF00248432)

**IMPORTANT NOTE:** You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

*Document Version*  
Publisher's PDF, also known as Version of record

*Publication date:*  
1990

[Link to publication in University of Groningen/UMCG research database](#)

*Citation for published version (APA):*

DOUMA, A. C., VEENHUIS, M., SULTER, G. J., WATERHAM, H. R., VERHEYDEN, K., MANNAERTS, G. P., & HARDER, W. (1990). PERMEABILITY PROPERTIES OF PEROXISOMAL MEMBRANES FROM YEASTS. *Archives of Microbiology*, 153(5), 490-495. DOI: 10.1007/BF00248432

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## Permeability properties of peroxisomal membranes from yeasts

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Received September 20, 1989/Accepted December 27, 1989

**Abstract.** We have studied the permeability properties of intact peroxisomes and purified peroxisomal membranes from two methylotrophic yeasts. After incorporation of sucrose and dextrans in proteoliposomes composed of asolectin and peroxisomal membranes isolated from the yeasts *Hansenula polymorpha* and *Candida boidinii* a selective leakage of sucrose occurred indicating that the peroxisomal membranes were permeable to small molecules. Since the permeability of yeast peroxisomal membranes in vitro may be due to the isolation procedure employed, the osmotic stability of peroxisomes was tested during incubations of intact protoplasts in hypotonic media. Mild osmotic swelling of the protoplasts also resulted in swelling of the peroxisomes present in these cells but not in a release of their matrix proteins. The latter was only observed when the integrity of the cells was disturbed due to disruption of the cell membrane during further lowering of the concentration of the osmotic stabilizer. Stability tests with purified peroxisomes indicated that this leak of matrix proteins was not associated with the permeability to sucrose. Various attempts to mimic the in vivo situation and generate a proton motive force across the peroxisomal membranes in order to influence the permeability properties failed. Two different proton pumps were used for this purpose namely bacteriorhodopsin (BR) and reaction center-light-harvesting complex I (RCLH<sub>1</sub> complex). After introduction of BR into the membrane of intact peroxisomes generation of a pH-gradient was not or barely detectable. Since this pump readily generated a pH-gradient in pure liposomes, these results strengthened the initial observations on the leakiness of the peroxisomal membrane

fragments. Generation of a membrane potential ( $\Delta\psi$ ) was also not observed when RCLH<sub>1</sub> complex was introduced into vesicles of purified peroxisomal membranes. The significance of the observed permeability of isolated yeast peroxisomal membranes to small molecules with respect to current and future in vitro import studies is discussed.

**Key words:** *Hansenula polymorpha* – *Candida boidinii* – Peroxisome – Peroxisomal membrane – Permeability – Membrane fusion

In yeasts the development and enzymic composition of microbodies (peroxisomes, glyoxysomes) can be largely prescribed by manipulating growth conditions (Zwart 1983; Veenhuis and Harder 1987, 1990). This makes these organisms suitable model systems for investigations into the molecular mechanisms of the biogenesis of microbodies. However, one of the main impediments in the progress of the current studies in this area is that an efficient in vitro protein import system as is known for other cell organelles (like mitochondria and chloroplasts), is not yet available. In vitro import has so far only been reported for acyl CoA oxidase in microbodies isolated from alkane-utilizing yeast (Small et al. 1988), although the efficiency of this system is low. In comparable experiments, conducted with peroxisomes isolated from the methylotrophic yeast *Hansenula polymorpha*, association of precursors of matrix proteins with the organelles was observed, but no evidence for import was obtained (A. C. Douma, unpublished results). The reason for this is not yet clear. Recent experiments of Goodman and coworkers (Bellion and Goodman 1987; cf. J. M. Goodman in: Borst 1989) suggested that under in vivo conditions import and assembly of peroxisomal matrix enzymes in methanol-grown *Candida boidinii* is dependent on the energy status of the organelles. Therefore, the failure or low efficiency of an in vitro import system may be due to insufficient energization of the membrane of

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*Abbreviations:* CL, cardiolipin; PE, phosphatidylethanolamine; PC, phosphatidylcholine; MES, 2-(N-Morpholino)ethanesulfonic acid; R<sub>18</sub>, octadecyl Rhodamine B Chloride; SUVs, small unilamellar vesicles; RCLH<sub>1</sub>-complex, reaction center-light-harvesting complex I; BR, bacteriorhodopsin; DCCD, N,N'-dicyclohexylcarbodiimide

isolated peroxisomes under the experimental conditions employed. This, in turn, may be related to the permeability properties of isolated peroxisomes. Recent work by Van Veldhoven et al. (1983, 1987) revealed that peroxisomal membranes isolated from rat liver are permeable to a range of small organic molecules; these workers and others suggested that this *in vitro* permeability is associated with the presence of a — possibly voltage-dependent — pore forming protein (Labarca et al. 1986) in the peroxisomal membrane. These observations prompted us to investigate the permeability properties of peroxisomal membranes purified from yeasts. The results of these studies are reported in this paper.

## Materials and methods

### *Organisms and cultivation*

*Hansenula polymorpha* de Morais et Maya CBS 4732 and *Candida boidinii* ATCC 32195 were grown in a continuous culture at 37°C and 30°C, respectively, at a dilution rate of 0.07–0.1 h<sup>-1</sup> in the medium described previously (Douma et al. 1985) using 1% (w/v) methanol as the source of carbon and energy.

### *Preparation of protoplasts*

Protoplasts were prepared as described by Douma et al. (1985). In osmotic shock experiments performed with *H. polymorpha* the sorbitol concentration in the protoplast suspensions was lowered stepwise from the initial value of 3.0 M to a final concentration of 2.5, 2.0, 1.5, 1.0 and 0.5 M sorbitol, respectively, by the addition of 50 mM potassium phosphate buffer pH 7.5 containing 1 mM MgCl<sub>2</sub> and 1 mM EDTA.

### *Cell fractionation*

Protoplasts of methanol-grown *H. polymorpha* were washed in buffer A (5 mM 2-(N-Morpholino)ethanesulfonic acid (MES) containing 1 mM MgCl<sub>2</sub> and 1 mM EDTA) to which 3 M (final concentration) sorbitol was added, then resuspended in buffer A containing 1 M sorbitol and homogenized in a Potter Elvehjem homogenizer (Potter 1955) by applying 2 strokes at 200 rpm. Immediately after homogenization the sorbitol concentration was increased to 2 M by adding an equal volume of 3 M sorbitol to buffer A and the homogenate was differentially centrifuged as described by Douma et al. (1985). Sucrose gradient centrifugation was performed by the method of Douma et al. (1987).

Peroxisomes from *C. boidinii* were isolated by a similar procedure (Goodman 1985).

Membranes of isolated peroxisomes were obtained using the floatation method described by Goodman et al. (1986). After floatation the membranes were collected, pelleted, resuspended in 20 mM Hepes/KOH buffer, pH 7.0 and stored in liquid nitrogen.

Protein concentrations were measured with the method of Bradford (1976) using bovine serum albumin as a standard.

### *Preparation of liposomes*

Small unilamellar vesicles (SUVs) were prepared by sonication under an atmosphere of nitrogen gas at 0°C.

For fusion experiments with intact peroxisomes SUVs composed of 10 mg/ml phosphatidylcholine (PC)/cardiolipin (CL) (molar ratio 1:1), were prepared in 5 mM MES-buffer pH 5.8 + 1 mM MgCl<sub>2</sub> + 1 mM EDTA + 50% (w/w) sucrose (buffer B). Bacteriorhodopsin (BR) containing SUVs composed of PC and CL were prepared in buffer B as described by Driessen et al. (1985a).

For fusion experiments with peroxisomal membranes reaction center-light-harvesting complex I (RCLH<sub>1</sub> complex) containing liposomes composed of PE were prepared in buffer A as described by Molenaar et al. (1988).

Liposomes were fused with isolated peroxisomes by the low pH-induced fusion method described by Driessen et al. (1985b). Incubation was for 30 min at 30°C in buffer B using a 10-fold excess of liposome phospholipid compared to peroxisome phospholipid (peroxisomal phospholipid/protein ratio's based on data from rat liver; Fujiki et al. 1982). After fusion of BR-containing liposomes with isolated peroxisomes from methanol-grown *H. polymorpha*, fusion products and liposomes were separated by floatation in a sucrose gradient as described previously (Douma et al. 1987).

Liposomes were fused with peroxisomal membranes by freeze-thawing (Crielaard et al. 1988).

### *Fusion assay*

For monitoring fusion, SUVs containing 4 mol% octadecyl Rhodamine B Chloride (R<sub>18</sub>) were used; relief of self-quenching of the fluorescence of this compound upon fusion, using equal amounts of liposome-phospholipid and peroxisome-phospholipid, was determined as described by Hoekstra et al. (1984). The fluorescence was expressed as a percentage of the fluorescence determined in controls to which 0.5% Triton was added.

### *Permeability measurements*

Asolectin liposomes were prepared as described by Van Veldhoven et al. (1987). The (sucrose-)permeability of peroxisomal membranes of *C. boidinii* and *H. polymorpha* was determined by the method of Van Veldhoven et al. (1987). Sucrose leakage is expressed as normalized [<sup>3</sup>H]dextran/[<sup>14</sup>C]sucrose ratios retained in the (proteo)liposomes (Van Veldhoven et al. 1987).

### *Fluorescence quenching studies*

9-Aminoacridine fluorescence quenching was measured at room temperature with a Perkin Elmer 204 spectrofluorimeter MPF-44B at 455 nm after excitation at 405 nm. The reaction mixture (2 ml) contained 5 mM MES pH 5.8, 1 mM MgCl<sub>2</sub>, 1 mM EDTA, 50% (w/w) sucrose, 0.5 μM 9-aminoacridine, 50 μl BR-containing liposomes or an equivalent amount of fusion products. The reaction was started by illumination of the reaction mixture with light with a wavelength higher than 570 nm.

### *Measurement of ΔΨ*

The ΔΨ generated by RCLH<sub>1</sub> complexes was determined by measuring the distribution of tetraphenylphosphonium ions (TPP<sup>+</sup>) across the liposomal membranes using a TPP<sup>+</sup> sensitive ion-selective electrode (Molenaar et al. 1988).

### *Electron microscopy*

Suspensions of protoplasts and isolated peroxisomes were prepared for electron microscopy as described before (Douma et al. 1987).

**Table 1.** Sucrose permeability of yeast peroxisomal membranes

A. Proteoliposomes composed of asolectin and peroxisomal membranes	
<i>C. boidinii</i>	4.56 ± 0.15
<i>H. polymorpha</i>	7.74 ± 0.46
B. Proteoliposomes composed of phosphatidylethanolamine and peroxisomal membranes	
<i>C. boidinii</i>	3.69 ± 0.14
<i>H. polymorpha</i>	3.80 ± 0.23
C. Peroxisomal membrane vesicles	
<i>C. boidinii</i>	4.70
<i>H. polymorpha</i>	8.24
D. Liposomes	
asolectin liposomes	1.05 ± 0.03
PE-liposomes	1.40 ± 0.10

Normalized ratios of [<sup>3</sup>H]dextran/[<sup>14</sup>C]sucrose retained in proteoliposomes containing peroxisomal membrane fragments were determined in the permeability assay described by van Veldhoven et al. (1987). Yeast peroxisomal membranes (approximately 22 µg of protein) were fused with (A) asolectin-liposomes (1.6 mg of phospholipid) or (B) PE-liposomes (0.25 mg of phospholipid) (*n* = 4). Also unfused yeast peroxisomal membranes (150 µg of protein) were tested (C; *n* = 1). Pure asolectin liposomes (1.6 mg of phospholipid) or PE-liposomes (0.25 mg of phospholipid) were used as a control (D; *n* = 4).

The subcellular localization of alcohol oxidase activity was demonstrated by the method of Veenhuis et al. (1976).

For freeze-etching isolated peroxisomes were incubated in the osmotically stabilized medium in which they were isolated supplemented with 10% (v/v) glycerol for 5 min, frozen in liquid FREON, stored in liquid nitrogen and subsequently freeze-fractured in a Balzer's freeze-etch unit according to Moor (1964).

## Results

### Permeability measurements

The permeability properties of peroxisomal membranes were studied by measuring dextran over sucrose ratios by similar methods as described before for rat liver peroxisomes (Van Veldhoven et al. 1987). To this purpose peroxisomal membranes, isolated from methanol-grown *Candida boidinii* and *Hansenula polymorpha*, were fused with asolectin liposomes to form proteoliposomes, simultaneously loaded with [<sup>3</sup>H]dextran and [<sup>14</sup>C]sucrose. The leakage ratios, summarized in Table 1, show that under these conditions a high specific leakage of sucrose occurred from proteoliposomes containing peroxisomal membranes of either of both yeasts studied (Table 1A). In pure asolectin liposomes used as a control no leakage was found (Table 1D) indicating that the observed permeability to sucrose was due to the presence of peroxisomal membrane fragments. In an additional experiment liposomes of another phospholipid namely PE were used. The sucrose leakage observed in these experiments did not differ significantly from the data for asolectin-containing proteoliposomes (Table 1B, D). Similar results were obtained after incorporation of both sugars in purified peroxisomal membrane vesicles solely (Table 1C).

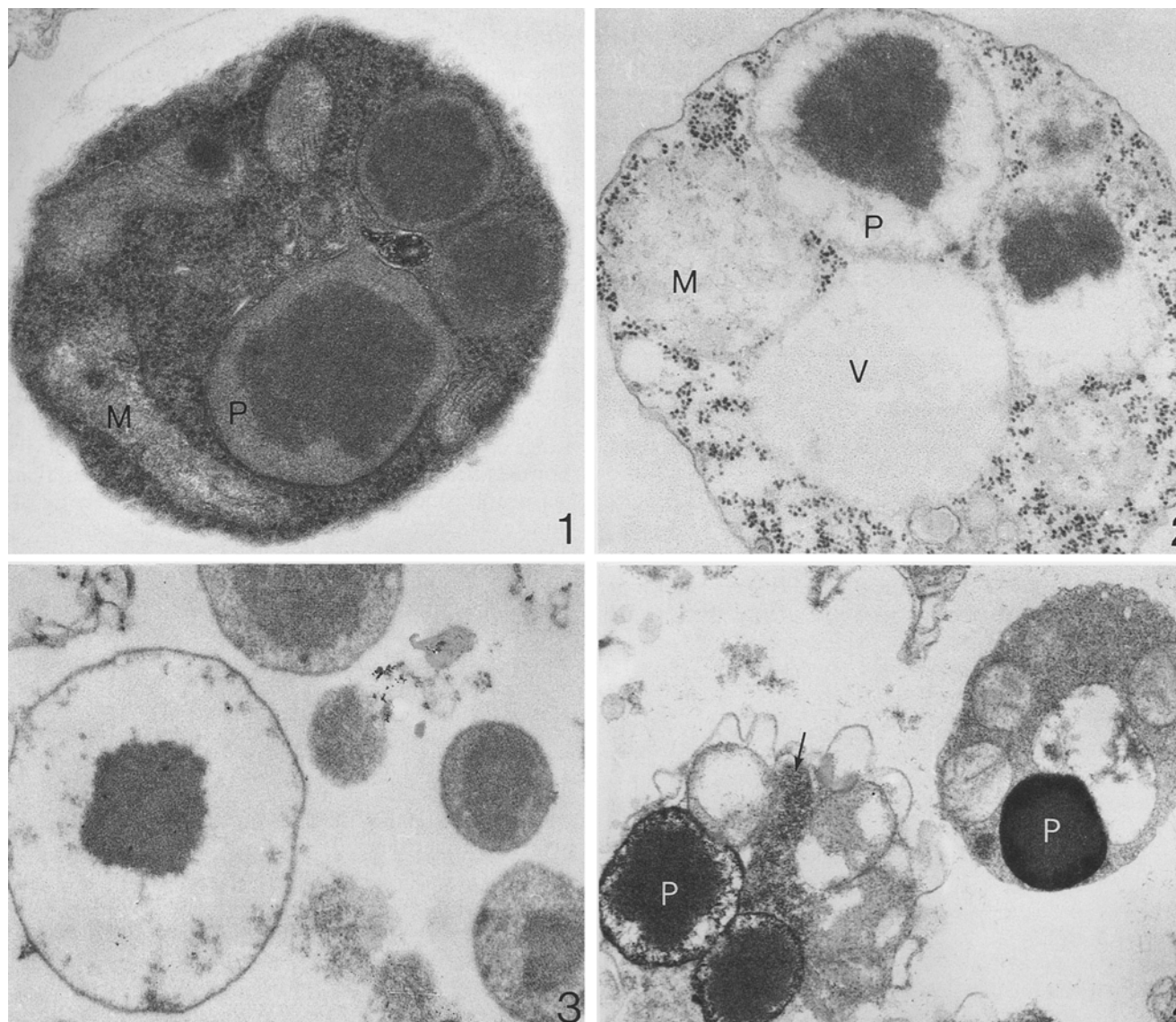
In all cases the membranes formed vesicles as was demonstrated by electron microscopy (data not shown).

### Stability of peroxisomes

Since in vivo a pH-gradient exists across peroxisomal membrane of yeasts (Nicolay et al. 1987), indicating that the membrane is not freely permeable under these conditions, the observed permeability in vitro may be due to a — possibly irreversible — damage of this membrane as a result of organelle isolation. Alternatively, it may be caused by a voltage-regulated pore-forming protein, as suggested for rat liver peroxisomes (Labarca et al. 1986; Van Veldhoven et al. 1987). The first possibility was studied by investigating the sensitivity of the organelles to osmotic shock, a common step in the isolation procedure (Douma et al. 1987). In osmotically stabilized protoplasts from both yeasts studied the peroxisomes are shown to have the size and matrix density seen in intact whole cells (Fig. 1) and all stain positively for different peroxisomal enzyme activities (Veenhuis et al. 1978). After mild osmotic swelling of protoplasts of *H. polymorpha* (by reducing the sorbitol concentration from 3 to 2 M), swelling of the majority of peroxisomes also occurred (Fig. 2); cytochemically the leakage of alcohol oxidase (compare Fig. 4) or catalase was not observed. Leakage of at least part of the soluble matrix proteins only occurred when the integrity of the protoplasts was disturbed due to disruption of the cell membrane during further lowering of the sorbitol concentration (Figs. 3, 4). The observed osmotic swelling of peroxisomes may persist after isolation of the organelles (Fig. 3). However, the leakage of matrix proteins observed during cell disruption did not continue after purification of the organelles by sucrose density gradient centrifugation. This was indicated by the finding that leakage of catalase was not observed from freshly isolated organelles from either *H. polymorpha* or *C. boidinii*, kept at 0°C under conditions where they are most stable (pH 5.8; Goodman et al. 1984), in the initial 3 h after isolation (data not shown). These results indicate that (i) leakage of matrix proteins during organelle isolation and the sucrose permeability of yeast peroxisomal membranes probably are not correlated and (ii) the protein leak is an instant process most probably associated with disturbance of the integrity of the cytoplasm.

### Liposome-mediated introduction of a foreign proton pump into the membrane of intact peroxisomes and isolated peroxisomal membranes

As indicated above, the observed in vitro permeability may be related to the absence of a proton motive force across the peroxisomal membrane. For this reason we have sought for conditions to restore the pH gradient in the in vitro situation. However, the endogenous ATPase, present on the peroxisomal membrane (Douma et al. 1987), could not be used for this purpose. Firstly, the enzyme is relatively unstable (G. J. Sulter, unpublished results). A second major drawback is that the pH opti-



**Abbreviations:** M, mitochondrion; P, peroxisome; V, vacuole; N, nucleus. Cells and organellar fractions are from methanol-grown *H. polymorpha*, fixed in glutaraldehyde -  $\text{OsO}_4/\text{K}_2\text{Cr}_2\text{O}_7$  unless stated otherwise. **Figs. 1-4.** The effect of an osmotic shock on peroxisome stability. **Figs. 1, 2.** Survey of an osmotically stabilized (Fig. 1;  $\times 46,000$ ) and a swollen protoplast (Fig. 2;  $\times 51,000$ ) showing the difference in matrix density of the peroxisomes. **Fig. 3.** Ultrathin section through a 1:1 mixed sample of osmotically swollen and

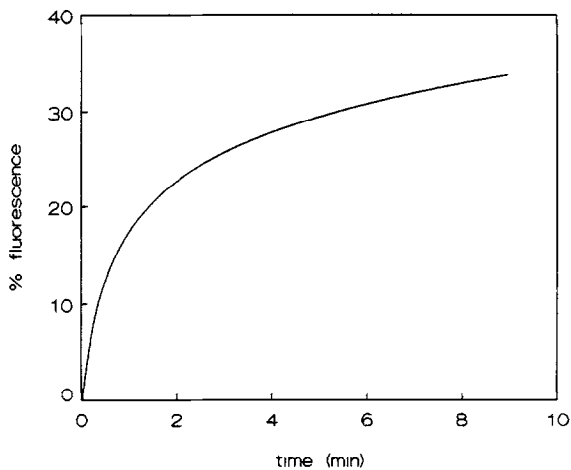
osmotically stabilized isolated peroxisomes (organelle at right hand side) demonstrating the organellar volume increase (organelle at left hand side) that may occur ( $\times 45,000$ ). **Fig. 4.** Leakage of the soluble part of alcohol oxidase from peroxisomes in protoplasts with a disrupted plasma membrane due to osmotic shock (left hand side, arrow), but not from peroxisomes in intact protoplasts (right hand side). Osmotically shocked protoplasts were mixed 1:1 with untreated cells prior to incubation with  $\text{CeCl}_3$  and methanol ( $\times 26,000$ )

mum of this ATPase is 8.5 (Douma et al. 1987), conditions where yeast microbodies are highly unstable (Goodman et al. 1984; Veenhuis et al. 1986). Therefore, we investigated whether as alternatives foreign proton pumps could be used.

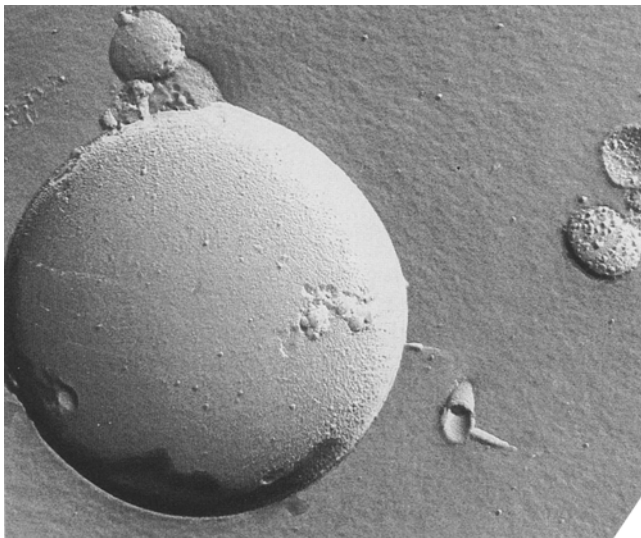
As a first approach we studied whether BR could be introduced into the membrane of intact peroxisomes by means of fusion of these organelles with BR-containing liposomes. After incubation of peroxisomes isolated from methanol-grown *H. polymorpha* with such liposomes at a low pH (Driessen et al. 1985 b) fusion indeed occurred. Firstly, fusion was indicated by the relief of self-

quenching of the fluorescent probe  $\text{R}_{18}$  which had been incorporated in the liposomal membrane vesicles (Fig. 5). Secondly, fusion was confirmed electron microscopically by freeze-etch methods (Fig. 6).

Fluorescence quenching studies showed that in suspensions of such fusion products light-induced quenching of the fluorescence of 9-aminoacridine takes place immediately after fusion. However, after separation of fusion products from unfused liposomes in a sucrose gradient generation of a pH-gradient was hardly detectable. In the same time interval the capacity of unfused



**Fig. 5.** Fluorescence development after incubation of CL/PC liposomes containing 4 mol% R<sub>18</sub> with isolated peroxisomes from methanol-grown *H. polymorpha*



**Fig. 6.** Freeze-etch replica demonstrating the presence of protein particles (BR) in the peroxisomal membrane ( $\times 78,000$ ). In normal (unfused) peroxisomal membranes such particles are invariably absent (van Dijken et al. 1975)

BR-containing liposomes to generate a pH-gradient was not significantly affected.

Comparable results were obtained when a different proton pump and purified peroxisomal membranes were used in these experiments. For instance, after fusion of peroxisomal membranes from *C. boidinii* and liposomes containing RCLH<sub>1</sub> complexes, generation of a membrane potential ( $\Delta\Psi$ ) was also not observed. In addition, experiments performed in the presence of DCCD, which is supplemented to block a possible proton leak due to disrupted ATPase (Linnett and Beechey 1979; Schneider and Altendorf 1987) and/or divalent anions ( $Mg^{2+}$  or  $Ca^{2+}$ ) to close a possible pore regulated by these ions did not alter the results.

## Discussion

The results, presented in this study, led us to the following conclusions: (i) yeast peroxisomal membranes are permeable to sucrose in vitro; (ii) the observed sucrose permeability is not correlated with the leak of part of the matrix protein, a common phenomenon of the isolation procedure of yeast peroxisomes (Veenhuis et al. 1986); (iii) the permeability properties prevented a proper restoration of the original in vivo energization of the peroxisomal membrane under the in vitro conditions employed.

Taken together our data suggest that yeast peroxisomal membranes in this respect behave as peroxisomal membranes from rat liver (Van Veldhoven et al. 1983, 1987). The latter membranes are also permeable to a range of other low molecular weight compounds. In vivo, however yeast peroxisomal membranes are probably not permeable, since a pH-gradient of approximately 1.2 pH-units exists across their membrane (Nicolay et al. 1987). This gradient is generated by a proton-translocating ATPase (Douma et al. 1987, 1990a), rendering the organelles internally acid (pH 5.8) (Nicolay et al. 1987) with respect to the cytosol.

In isolated rat liver peroxisomes leakage has been ascribed to the presence of a peroxisomal membrane protein of low molecular weight, which was thought to be a pore-forming protein (Van Veldhoven et al. 1983, 1987). The presence of a possibly voltage-dependent weakly cation-selective pore in rat liver peroxisomal membranes was confirmed in studies by Labarca et al. (1986). Similarly, also yeast microbody membranes may contain a pore-forming protein. Preliminary experiments, using antibodies against the 31,000 D pore-forming protein of the mitochondrial outer membrane of *Saccharomyces cerevisiae* (De Pinto et al. 1987) indicated that these antibodies crossreacted with a protein of identical mass, present in highly purified peroxisomal membranes of both baker's yeast and *H. polymorpha* (W. H. Kunau and M. Veenhuis, unpublished results). These observations add to our hypothesis that in yeast peroxisomal membranes also a regulatable pore-forming protein may be present. We are currently investigating the occurrence and properties of such a protein in detail.

The present situation that yeast peroxisomes are invariably leaky after their isolation has a major impact on current studies on peroxisome biogenesis in that it does not allow to investigate a possible energy-dependency of import of matrix proteins in an in vitro system. The need for this is indicated by recent work of Bellion and Goodman (1987) who demonstrated that in vivo import of peroxisomal matrix proteins in methanol-grown *C. boidinii* is prevented by uncouplers. This effect also occurred at low uncoupler-concentrations, where the ATP-level in the cell was not greatly affected (cf. J. M. Goodman in: Borst 1989), thus indicating the necessity of a proton motive force for the import of peroxisomal proteins. On the other hand, in vitro uptake of acylCoA oxidase in isolated rat liver peroxisomes was shown to be ATP-dependent but, as expected from the known leakiness of the organelles, not prevented by uncouplers

(Imanaka et al. 1987). However, in yeasts comparable in vitro import assays showed very low efficiencies (Small et al. 1988) or failed (A. C. Douma, unpublished results). Therefore, yeast peroxisomes isolated by the current established procedures may not provide optimal starting material for the development of an efficient in vitro protein system. Until improved isolation procedures are available and/or the mechanism regulating the putative pore is known, an in vivo protein import system (Douma et al. 1990b) may be a useful alternative in the study of peroxisome biogenesis.

**Acknowledgements.** Thanks are due to Arnold Driessen for his interest and valuable suggestions, to Wim Crielaard for a gift of RCLH<sub>1</sub> containing liposomes and to Klaas Sjollem and Jan Zagers for skillful assistance in different parts of this study. Anneke Douma and Hans Waterham are supported by The Netherlands Technology Foundation (STW) and Grietje Sulter by the Foundation for Fundamental Biological Research (BION) which are subsidized by The Netherlands Organization for the Advancement of Pure Research (NWO).

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