Peroxisomes and Ether Lipid Biosynthesis in Rat Testis and Epididymis

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ABSTRACT

Plasmalogens are a main component of the spermatozoon membrane, playing a crucial role in their maturation. The initial steps in plasmalogen biosynthesis are catalyzed by two peroxisomal enzymes, dihydroxyacetonephosphate acyltransferase and alkyl-dihydroxyacetonephosphate synthase. The localization of both enzymes in the membrane of peroxisomes implies that plasmalogen-producing cells should contain this organelle. To unravel the putative source of spermatozoan plasmalogens we investigated which cell types in the testis and epididymis are endowed with peroxisomes. To this extent, testicular and epididymal tissue was analyzed at the protein and RNA levels by means of light and electron microscopical immunocytochemistry as well as by Western and Northern blotting. Proteins and mRNAs of peroxisomal enzymes, especially those of dihydroxyacetonephosphate acyltransferase and alkyl-dihydroxyacetonephosphate synthase, were detected in the testis and epididymis. In the testis, peroxisomes were localized exclusively in Leydig cells and not in cells of the seminiferous tubules, implying that the latter do not contribute to the biosynthesis of plasmalogens of the sperm membrane. In contrast, peroxisomes could be clearly visualized in the epithelial cells of the epididymis. The results suggest that peroxisomes in epithelial cells of the rat epididymis play a pivotal role in the biosynthesis of plasmalogens destined for delivery to the sperm plasma membrane.

epididymis, Leydig cells, male reproductive tract, sperm maturation, testis

INTRODUCTION

The plasma membrane in the anterior head region of a mature sperm cell has several distinguishing features that affect membrane stability and may be adapted to the requirements of the acrosome reaction. One of the most striking attributes is the high content of a unique species of choline phospholipids that contain an ether-linked fatty alcohol at position one of the glycerol moiety and a highly unsaturated acyl chain at position two [1]. These glycerolipids, which are also referred to as plasmalogens [2], are reported to account for the unique fusogenic potential of sperm plasma membrane domains [1, 3, 4]. In addition, they may also play a direct role in protecting animal cell membranes against oxidative stress [5–9].

The initial steps of plasmalogen biosynthesis are catalyzed by dihydroxyacetone phosphate (DHAP) acyltransferase (DHAP-AT) and alkyl-DHAP synthase (DHAP-S), both of which have been localized in the membranes of peroxisomes [10–12]. DHAP-AT catalyzes the transesterification of DHAP to form acyl-DHAP by utilizing long-chain acyl coenzyme As (CoAs) as acyl donors. DHAP-S then replaces the acyl chain in acyl-DHAP by a long-chained fatty alcohol, thus forming alkyl-DHAP, which is the first detectable intermediate in ether lipid biosynthesis [2]. Subsequent processing of alkyl-DHAP is directed by enzymes localized to the endoplasmic reticulum (ER) [4]. Because DHAP-AT and DHAP-S catalyze the initial steps in ether lipid biosynthesis and, moreover, are supposedly confined to peroxisomes [11, 12], this organelle appears to be indispensable for plasmalogen biosynthesis. This notion is supported by the finding that tissues and cells from patients with Zellweger syndrome, who are known to lack functional peroxisomes, are severely deficient in ether phospholipids and in the enzymes DHAP-AT and DHAP-S [12, 13].

Peroxisomes are single membrane-bound organelles that are almost ubiquitous in eukaryotic cells. In mammals, peroxisomes contain more than 50 different enzymes with important roles in various metabolic pathways [14]. Moreover, they house oxidases, which produce hydrogen peroxide, and catalase, which degrades it [15]. In addition to their involvement in plasmalogen biosynthesis, they are engaged in the β-oxidation of very-long-chain fatty acids [16] and prostaglandins [17], and the synthesis of cholesterol [18–20].

The peroxisomal localization of the enzymes that are indispensable to ether lipid biosynthesis implies that cells producing plasmalogens must contain peroxisomes. Spermatozoa however, do not contain this organelle and, in addition, the epithelium of testicular tubules was reported earlier to be devoid of peroxisomes [21, 22]. Thus, spermatozoa may be provided with plasmalogens after having left the testis. Indeed, the plasmalogen content of the sperm plasma membrane increases during sperm passage through the epididymis [3], which strongly suggests that this part of the reproductive tract accounts for the synthesis of sperm ether lipids.

The present study was intended to identify the epididymal cells housing peroxisomes and hence providing spermatozoa with plasmalogens, and to reinvestigate the occurrence of the organelle in testis. To this end, we have used Western and Northern blotting in combination with immunocytochemistry to study the expression of peroxisomal enzymes both at the protein and RNA levels.

MATERIALS AND METHODS

Tissue Preparation

Male Sprague-Dawley rats weighing 200–250 g were maintained on a standard laboratory diet. The testes and epididymides were removed under anesthesia (pentobarbital). One portion of each sample was immediately frozen in liquid nitrogen for subsequent biochemical analyses. For light microscopical immunohistochemical studies, samples were fixed in Carnoy fixative containing 60% ethanol, 30%
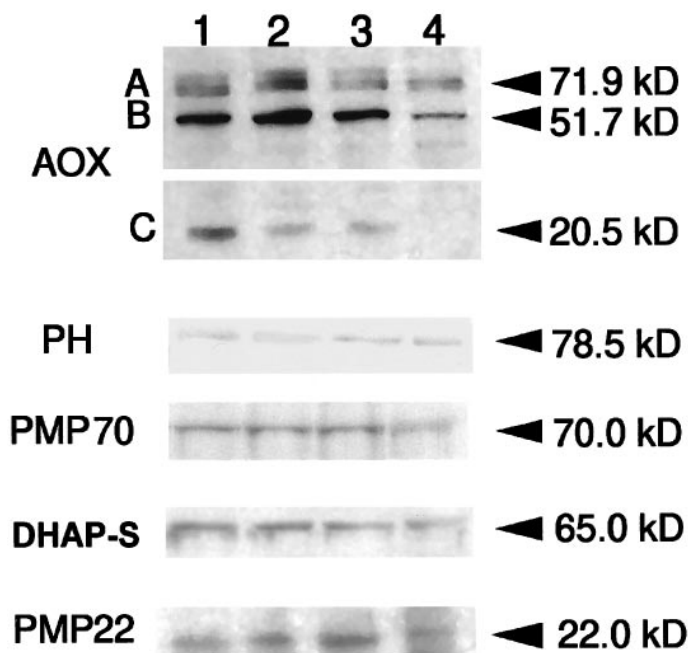
FIG. 1. Immunoblots of different sections of epididymis (lane 1, caput; lane 2, corpus; lane 3, cauda) and testis (lane 4). Equal amounts of protein (10 μg) were applied to each lane. Blots were incubated with antibodies to AOX, PH, PMP70, DHAP-S, and PMP22. The immunocomplexes were visualized by the ECL technique (for details see *Materials and Methods*).
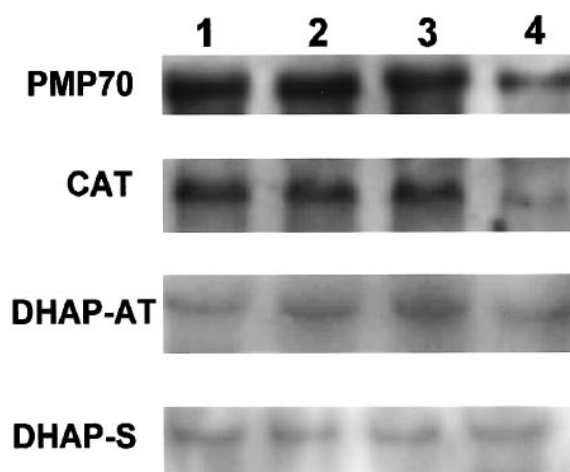

FIG. 2. Northern blots. Equal amounts of RNA isolated from different sections of epididymis (lane 1, caput; lane 2, corpus; lane 3, cauda) and testis (lane 4) were loaded on each lane. The blots were incubated with cRNA probes for PMP70, CAT, DHAP-AT, and DHAP-S.

chloroform, and 10% glacial acetic acid [23]. Tissue slices were dehydrated in a graded series of ethanol and embedded in paraffin (Paraplast Plus; Monoject Scientific, Athy, Ireland) at 57°C. For electron microscopical immunocytochemistry, samples were treated with a fixative containing 0.25% glutaraldehyde in 0.1 M PIPES buffer pH 7.4, followed by embedding in LR-white (London Resin Co. Ltd., Hampshire, U.K.).

Antibodies, SDS-PAGE, and Immunoblotting

Polyclonal antibodies were raised against DHAP-S; catalase; 70-kDa and 22-kDa peroxisomal membrane proteins (PMP70 and PMP22); and the two peroxisomal β-oxidation enzymes, acyl-CoA oxidase (AOX) and enoyl-CoA-hydratase/3-hydroxyacyl-CoA-dehydrogenase/3,2-enoyl-CoA-isomerase (PH), also referred to as multifunctional enzyme [24]. The concentration of the antibodies used for Western blotting and immunocytochemistry was 1 μg protein/ml. Antibody specificity was assessed as described previously [25].

For Western immunoblotting, tissues were homogenized in a homogenization buffer (250 mM sucrose, 5 mM morpholino-propanesulfonic acid, 1 mM EDTA, 0.1% ethanol pH 7.4) using an Ultra-Turrax (IKA Labortechnik, Staufen, Germany) and equal amounts of protein were subjected to SDS-PAGE. After electrotransfer of the polypeptides onto nitrocellulose the sheets were incubated overnight with the antibodies described above. After repeated washings, a peroxidase-conjugated goat anti-rabbit antibody (Sigma, München, Germany) was added for 1 h at room temperature. The immunoreactive bands were visualized by enhanced chemiluminescence (ECL; Amersham International, Little Chalfont, Buckinghamshire, U.K.) according to the manufacturer's protocol.

Complementary DNA Cloning

Total RNA was isolated by means of the RNeasy mini kit (Qiagen, Hilden, Germany). Total RNA from rat liver and human HepG2 cells, as well as degenerated primers raised against published sequences of cavia DHAP-S and human DHAP-AT, were used to generate fragments of 160 base pairs (bp) and 310 bp of DHAP-S and DHAP-AT, respectively, by reverse transcriptase-polymerase chain reaction (RT-PCR; The Superscript One-Step RT-PCR System; Life Technologies International, Paisley, U.K.). The PCR fragments were cloned into *pCR*II-TOPO (Invitrogen, Groningen, The Netherlands) and sequenced to verify the correct insert.

Preparation of DIG-Labeled cRNA Probes

Digoxigenin (DIG)-labeled cRNA probes were prepared using a DIG RNA labeling kit (Roche, Mannheim, Germany). Briefly, the subcloned plasmids with inserts of rat liver PH, catalase, PMP70, DHAP-AT, and DHAP-S were amplified by PCR using pGEM or M13 oligonucleotides as primers. The amplified PCR products containing both SP6 and T7 promoter sequences were purified by low-melting agarose gel electrophoresis, extracted by the QIAquick gel extraction kit (Qiagen), and subsequently transcribed to obtain corresponding DIG-labeled cRNA probes. For transcription, 1 μg of template cDNA, 2 μl of either SP6 or T7 RNA polymerase, and 2 μl of a ribonucleotide mixture containing DIG-labeled UTP were suspended in 2 μl of 10× transcription buffer. Diethyl pyrocarbonate (DEPC) water was added up to 20 μl, and the mixture was incubated for 2 h at 37°C. Complementary RNA probes were precipitated at −20°C by 4 μl of 4 M LiCl, 2 μl of 0.5 M EDTA, and 75 μl of absolute ethanol. The precipitate was collected for 15 min at 13 000 × *g*, washed with 70% ethanol, dried, and resuspended in 50 μl of DEPC water. Discrimination of antisense and sense transcripts as well as assessment of the sensitivity of the probes were accomplished by Northern blotting.

Northern Blot Analysis

Northern blotting was performed as described previously [26]. Total RNA was prepared from the indicated tissues

using either the Roti-Quick-Kit (Roth, Karlsruhe, Germany) or the RNeasy mini kit (Qiagen) and separated by electrophoresis on a formaldehyde-agarose gel. Up to 20 μg of total RNA were loaded per lane. RNAs were transferred onto a nylon membrane (Biodyne A; Pall, Dreieich, Germany) by capillary transfer, immobilized by ultraviolet irradiation (Stratalinker R 2400; Stratagene, La Jolla, CA), and prehybridized according to the manufacturer's protocol (*The DIG System User's Guide,* provided by Roche). For hybridization, 100 ng/ml of the corresponding antisense cRNA probe were added to the prehybridization mixture, and incubation of the membranes was continued at 68°C overnight. Thereafter, it was washed twice for 5 min at room temperature in 2× standard saline-sodium citrate (SSC) buffer/0.1% SDS, and twice for 25 min at 68°C in 0.1× SSC/0.1% SDS. Nonhybridized, single-strand RNA was subsequently removed by RNase A digestion. For this, the membrane was soaked for 10 min at room temperature in digestion buffer (10 mM Tris-HCl pH 7.5, 5 mM EDTA, 300 mM NaCl), 1 μg/ml of RNase A was added, and incubation was continued for another 10 min. To stop RNase A digestion and to remove RNA fragments, the membrane was incubated for 10 min in digestion buffer devoid of RNase A, followed by a wash with 2× SSC/0.1% SDS for 30 min at room temperature. DIG-labeled RNA hybrids were immunocomplexed with an alkaline phosphatase-labeled anti-DIG antibody, and the complexes were visualized by ECL with CDP-Star serving as substrate (Roche).

Morphological Localization of Peroxisomes

Light microscopy. Paraffin sections were incubated for immunohistochemistry using antibodies against CAT and PH. The bound antibodies were visualized with a biotinylated secondary antibody followed by incubation with an avidin-peroxidase conjugate (ExtrAvidin peroxidase staining kit; Sigma) and aminoethylcarbazol as a substrate, according to the manufacturer's protocol. The sections were counterstained with hematoxylin.

Electron microscopy. Ultrathin sections of LR-white-embedded tissue were incubated with an antibody to CAT using the protein A-gold procedure with 12 nm gold particles [27].

RESULTS

Cloning of Rat DHAP-S and Human DHAP-AT Fragments

The 160-bp fragment of rat DHAP-S cloned as described before showed 94% homology to human DHAP-S and 93% homology to the corresponding guinea pig sequence. The novel sequence of the fragment, which has not been published so far, has been submitted to GenBank, accession number AF280054. The identity of the 310-bp fragment from human DHAP-AT was verified by sequencing.

Analysis of Peroxisomal Proteins and Messenger RNAs

Proteins of the peroxisomal matrix and membrane are expressed in the rat testis and the entire epididymis.

Protein. Peroxisomal marker proteins were identified in the testis, caput epididymidis, corpus epididymidis, and cauda epididymidis by Western blotting (Fig. 1). In line with earlier observations [25, 28, 29], AOX showed three distinct bands corresponding to molecular masses of 71.9 kDa, 51.7 kDa, and 20.5 kDa. Remarkably, these bands appeared in varying ratios in the different examined tissues. In the epididymidis, the 51.7-kDa band was very strong

compared with its rather weak appearance in the testis. The 20.5-kDa band was conspicuous only in the caput epididymidis and was less evident in the other tissues. The other peroxisomal markers investigated—PH (78.5 kDa); and PMP22, PMP70, and DHAP-S (65 kDa)—were detected in all tissues to quite comparable extents.

Messenger RNAs. The results of the mRNA analysis (Fig. 2) widely confirm the findings obtained by Western blotting. The testis and all parts of the epididymis contained the mRNAs for PMP70, catalase, DHAP-AT, and DHAP-S. Although the expressions of PMP70 and catalase were markedly stronger in the epididymis than in the testis, DHAP-AT and DHAP-S were uniformly distributed.

Morphological Observations

The immunostaining of light and electron microscopical sections showed identical staining with antibodies to different peroxisomal proteins (catalase, PH). In the testis (Fig. 3), immunoreactivity was restricted to the interstitial cells of Leydig; no labeling was detected within the seminiferous tubules. Electron microscopical immunocytochemistry of Leydig cells (Fig. 3c) revealed that the labeling was localized exclusively in the matrix of peroxisomes. The peroxisomes showed a circular shape and a profile diameter of 250–400 nm.

In light microscopical sections of the epididymis, immunoreactivity was observed in the ductuli efferentes and all regions of the ductus epididymidis except for the tall columnar epithelium of the initial segment. In Figure 4, a and b, a section of the corpus is shown revealing marked labeling in the basal part of the epithelium. By electron microscopical immunocytochemistry, peroxisomes were found in basal and in principal cells, frequently showing irregular profiles and a diameter of about 100 nm (Fig. 4c).

DISCUSSION

Up to now, peroxisomes in the epithelium of the epididymis have been widely ignored, and hence, data on their distribution in different cell types and their biological role are scanty. Moreover, the few reports published address exclusively the contribution of the organelle to peroxide metabolism without tackling more fundamental aspects; namely, the involvement of peroxisomal enzymes in the biosynthesis and degradation of lipids. Thus, Arrighi et al. [30] have described peroxisomes in the ductuli efferentes of domestic Equidae, suggesting that they may be involved in the oxidation and elimination of toxic peroxides generated during endocytotic and spermatophagic activities of the cells. Similarly, Zini and Schlegel [22, 31] have localized catalase mRNA in the rat epididymis, claiming that the enzyme supports epididymal function by maintaining a stable redox status in the organ. It was the intention of the present study to focus mainly on the putative ''lipid role'' of epididymal peroxisomes and here on their engagement in the ether lipid biosynthesis. The results obtained provide strong support that the production of plasmalogens may be a main function of those peroxisomes.

As outlined before, the initial enzymes of the ether lipid biosynthesis pathway, DHAP-AT and DHAP-S, are proteins of the peroxisomal membrane [10, 11], which require the presence of peroxisomes in cells and tissues in order to contribute to the biosynthesis of plasmalogens. Our Northern and Western blotting results indicate that peroxisomal proteins and mRNAs, especially those
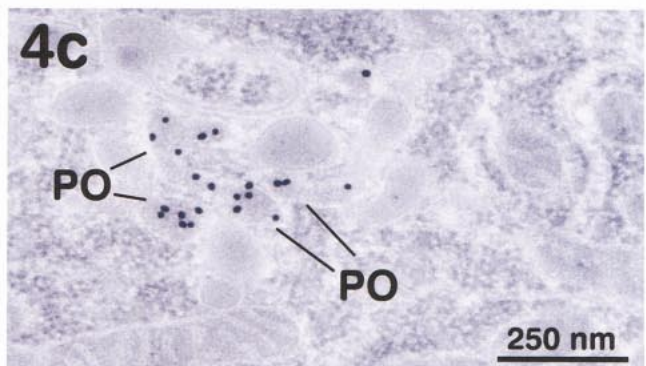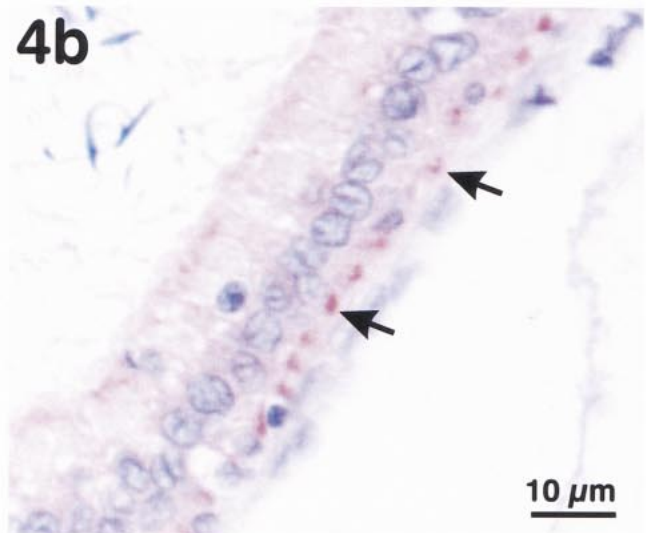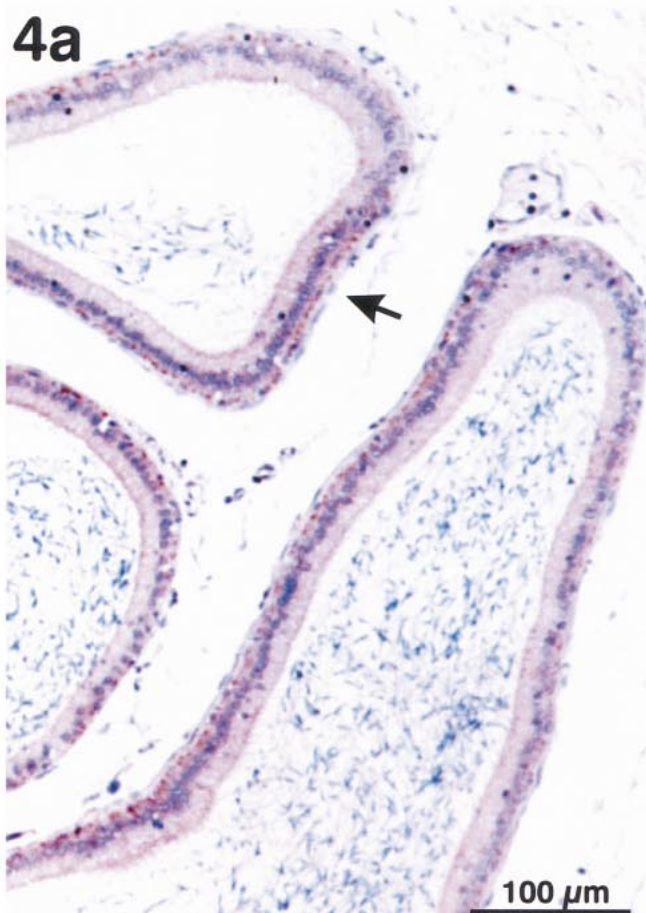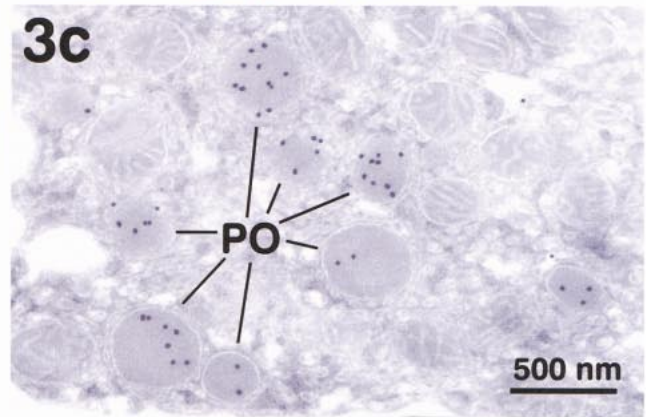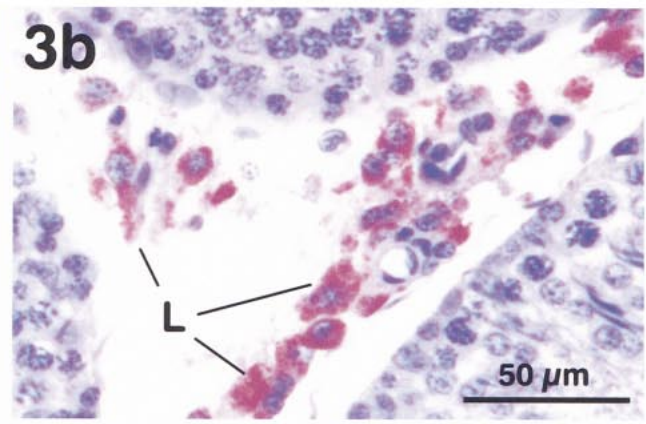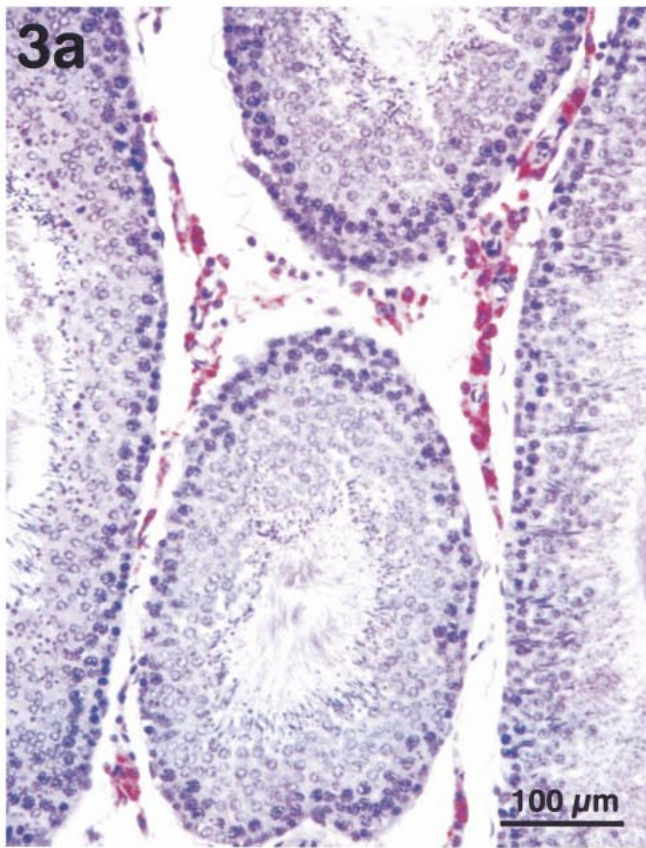
of DHAP-S and DHAP-AT, are present in both the epididymis and the testis. The morphological examination of the tissues, however, revealed that the testis is most probably not the source of the plasmalogens of the sperm plasma membrane because testicular peroxisomes could be detected only in the interstitial Leydig cells but not within the cells of the tubuli seminiferi. This corroborates earlier findings by Reddy and Svoboda [21] who described peroxisomes in isolated Leydig cells and recent reports on the localization of catalase mRNA by in situ hybridization in the same cell type but not within the seminiferous epithelium [22, 32]. In summary, these data provide strong evidence that peroxisomes may be at least very rare or even entirely absent in the spermatogenic as well as the Sertoli cells of the seminiferous tubules. Consequently, this implies that peroxisomes in the epithelial cells of the epididymis are the ones that are active in the biogenesis of plasmalogens destined for the sperm membrane. This notion is supported by the finding that indeed, the ether lipid content of the sperm membrane increases when a spermatozoon passes through this part of the male reproductive tract [1, 3].

The incorporation into the sperm plasma membrane of ''exogenous'' plasmalogens, which are produced by cells of the epididymis, raises intriguing questions regarding the mode of transport of the lipids across the epithelial cell and their delivery to spermatozoa. Based on the fact that the initial steps of plasmalogen biosynthesis occur in the membrane of peroxisomes while the succeeding steps proceed in the ER, it is reasonable to assume that alkyl-DHAP has to be translocated across the peroxisomal membrane for further conversions in the ER [2]. The manner in which the plasmalogens are transported to and incorporated into the sperm plasma membrane, however, is not yet fully understood, and various mechanisms are imaginable. The plasmalogens could be incorporated into the plasma membrane of the epithelial cells and transferred to the sperm cell membranes by direct contact. Another possible way could be that exocytosis of plasmalogens into the lumen of the ductus epididymidis. Recent studies on the incorporation and transmembrane movement of exogenous phospholipids in membranes of sperm cells [33], erythrocytes, and fibroblasts [34] indicate that the uptake and incorporation of plasmalogens into the sperm cell membrane may occur spontaneously. Nevertheless, an ATP-dependent mechanism may participate in the incorporation and especially in the movement from the outer to the inner leaflet [34]. Altogether, the massive alteration of the sperm plasma membrane composition during passage through the epididymis is a spe-cial phenomenon, and further work in this field may elucidate its benefit as a useful model for studying the uptake of exogenous phospholipid membrane components.

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FIG. 3. Immunocytochemical localization of peroxisomes in the testis. **3a** and **3b**) Light micrographs from sections incubated with an antibody to catalase. **3b**) Detail from **3a**. Note the specific (red) staining of the interstitial Leydig cells (L). **3c**) Electron micrograph from an interstitial Leydig cell incubated for immunocytochemical localization of catalase. PO, Peroxisomes.
FIG. 4. Immunocytochemical localization of peroxisomes in the epididymis (corpus region). **4a** and **4b**) Light micrographs from sections incubated with an antibody to PH. Similar staining was obtained with an antibody to catalase. The arrow in **4a** marks the area shown in **4b**. Note the specific (red) stained spots in the basal areas of the epithelium (some denoted by arrows in **4b**. **4c**) Electron micrograph from a basal area of a principal cell, incubated for immunocytochemical localization of catalase. The peroxisomes are flanked by various similar looking vesicles, which however, do not exhibit positive immunostaining.

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