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Immunocytochemical localization of peroxisomal proteins in human liver and kidney

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Summary: The sample preparation and immunocytochemical methods for investigating the presence and subcellular localization of peroxisomal proteins (catalase, the three β-oxidation enzymes, alanine:glyoxylate aminotransferase and a peroxisomal membrane protein) in human liver biopsies are described. We present a protocol for immunolabelling on ultrathin and semithin sections from the same tissue block, with protein A–colloidal gold as a reporter system. For this purpose, the tissue is embedded in Unicryl, a hydrophilic acrylic resin that is cured by ultraviolet illumination at 2°C. The limitations and possibilities of the methods are discussed together with methodological problems. Cryostat sections of prefixed material should be used for the visualization by light microscopy of cytoplasmic catalase. It is emphasized that immunolabelling for catalase in formalin-fixed archival liver samples and in liver autopsy tissue (in the latter also for the peroxisomal β-oxidation enzymes) permits visualization of peroxisomes; this can be helpful in diagnosing an index case retrospectively.

The impaired peroxisomal functions in patients with a peroxisomal disorder originate from the absence, mislocalization or deficient activity of one or more peroxisomal (enzyme) protein(s), or from the inability at the organelle level to assemble import-competent peroxisomes. The occurrence and subcellular localization of a protein can be determined by immunocytochemistry, which provides information about the tissue *in situ*. Here the method differs essentially from immunoblotting (see Wanders et al 1995), in which the presence of a protein is determined in tissue homogenates or in subcellular fractions. For the purpose of immunocytochemistry, the tissue architecture is kept as intact as possible via chemical fixation.

Several immunolocalization studies of peroxisomal matrix and membrane proteins have been performed in cultured skin fibroblasts from peroxisomal disorder patients. In the approach presented here, biopsied liver tissue is used. The argument is that cultured fibroblasts do not necessarily express the functional defect(s) observed in the patient (for recent examples see Mandel et al 1994; Schutgens et al 1994; Roels et al 1995a). In addition, peroxisomal enzymes may be expressed in the liver only, e.g. alanine:glyoxylate aminotransferase.

All immunocytochemical methods consist essentially of two main steps: (1) binding of the antibody to the antigen, based on the specific recognition of epitope(s) in the antigen against which the antibody is directed; (2) visualization of the bound antibody via a reporter system. The immunocytochemical methodology for the localization of catalase and the peroxisomal β-oxidation enzymes in human control liver and kidney at the light- and electron-microscope level was explored by Litwin et al (1987, 1988). They recommended protein A–colloidal gold as a detection method in combination with silver enhancement for light-microscopic visualization. The same detection system was used for the immunolocalization of catalase, the peroxisomal β-oxidation enzymes, alanine:glyoxylate aminotransferase and peroxisomal membrane proteins in the liver of peroxisomal disorder patients and in fetal human liver (Cooper et al 1988; Danpure et al 1989, 1993, 1994; Espeel et al 1990a,b, 1991a,b, 1993, 1995a,b; Hughes et al 1992, 1993) and kidney (Espeel et al 1991a).

Protein A occurs in the cell wall of almost all strains of *Staphylococcus aureus*. It has a high binding affinity for the Fc part of immunoglobulins belonging primarily to the IgG type. The binding affinity varies widely with the animal species from which the IgG is derived. All rabbit, pig, guinea pig and human IgGs are excellent protein A binders. Chicken IgG does not bind protein A and only a mild affinity is found for some mouse and rat IgG subclasses (Griffiths 1993).

Silver enhancement of colloidal gold particles is an application of the principle of 'autometallography' (Danscher and Noergaard, 1985; Danscher et al 1987). This principle is based on the property of several metals — including gold — to function as catalysts in the electron transfer for the reduction of silver ions to metallic silver. As a result, in the presence of an electron donor (hydroquinone), a shell of reduced (metallic) silver is deposited at the surface of the gold particles before silver precipitates in the medium. The metal particles visualize their own presence.

We describe here the procedures for immunolocalization at the light- and electron-microscope level of catalase (EC 1.11.1.6), the peroxisomal β-oxidation enzymes acyl-CoA oxidase (EC 1.3.99.3), bi(tri)functional enzyme (enoyl-CoA hydratase (EC 4.2.1.17 and 3-hydroxyacyl-CoA dehydrogenase (EC 1.1.1.35)) and 3-ketoacyl-CoA thiolase (EC 2.3.1.16), alanine:glyoxylate aminotransferase (EC 2.6.1.4) and a peroxisomal membrane protein (the latter at the ultrastructural level only) in liver samples embedded in Unicryl (BioCell, Cardiff, UK) — a recently introduced hydrophilic acrylic resin (Scala et al 1992) — from control subjects and from peroxisomal disorder patients. During the FEBS Advanced Course the protocol was demonstrated on the bench for the immunodetection of catalase in human control liver with polyclonal antibodies (IgG fraction) raised in rabbits against bovine liver catalase (Rockland Laboratories, code No. 200-4151). In Sections 1 and 2.4, several questions and remarks raised by the course participants are dealt with. The procedures for immunostaining for catalase and the peroxisomal β-oxidation enzymes at the light-microscope level (paraffin and cryostat sections) have been described previously (Litwin et al 1988; Espeel et al 1990a); they are not presented in this text, but the approach is dealt with in Section 2.4.

1. SPECIMEN PREPARATION

1.1. Fixation

The samples are fixed in 4% commercial formaldehyde in 0.12 mol/L sodium cacodylate (pH 7.3) containing 1% calcium chloride (w/v) at ambient temperature. The samples are sent to our laboratory by private express mail and, depending upon the duration of transport, the fixation time is usually around 24 h. For optimal tissue processing, it is preferred that the biopsy is taken and delivered in the first half of the week.

The choice of this fixative is determined by the fact that it is also used for the diaminobenzidine incubation to demonstrate catalase activity (see Roels et al 1995b) and that it is suited, according to Litwin et al (1987), for the immunocytochemical detection of the antigens mentioned in the introduction.

Upon arrival of the sample, small tissue pieces (less than 0.5 mm thick) are cut with a razor blade from the biopsy cylinder. These are further fixed in 0.5% glutaraldehyde in 0.1 mol/L sodium cacodylate (pH 7.3) containing 1% calcium chloride (w/v) for 1 h at 4°C. Thereafter they are rinsed in 0.1 mol/L sodium cacodylate (pH 7.3) containing 1% calcium chloride (w/v) at 4°C for at least 1 h. Depending on the time of arrival, they may be kept in this buffer at 4°C for an overnight period or over the weekend.

Remark: As a rule, part of the biopsy is divided into serial chopper sections while it is in the formaldehyde fixative. The sections are processed in alternating order for immunocytochemistry and for diaminobenzidine cytochemistry. The preparation of chopper sections implies that the tissue is enrobed in agar. The agar often sticks around the sections; it should be removed with a fine needle under a stereomicroscope before embedding in Unicryl.

1.2 Processing for Unicryl embedding

Specimens are rinsed for 1 h at 4°C in 0.1 mol/L sodium cacodylate (pH 7.3) without calcium chloride. The tissue samples are then immersed in ammonium chloride (50 mmol/L in phosphate-buffered saline (PBS, for preparation see Section 2.1) for 30 min at 4°C to block the free aldehyde groups. Dehydration follows at 4°C over ethanol at 50%, 70%, 90% (30 min each) and 100% (2×30 min). Specimens are then impregnated with pure Unicryl (2×1 h at 4°C); finally they are left in Unicryl overnight at 4°C. **Caution:** *Unicryl is supplied as a ready-to-use mixture. Handling unpolymerized Unicryl requires protective measures: wear acrylate-resistant gloves and work under an efficient fume hood.*

The next morning specimens are brought into BEEM capsules that are held in an aluminium rack. The rack is placed in an ultraviolet light polymerization chamber (Agar UVF 35; described by Glauert and Young 1989) at 2°C equipped with two UV lamps (Philips TL82/05; emitting at 360 nm). The distance between the capsules and the lamps is 10 cm. Polymerization takes 5 days.

1.3 Microtomy

Semithin sections (2 μm) are cut with a glass knife and mounted on silanated glass slides (see Section 3 for the preparation of silanated glass slides). Ultrathin sections (70–80 nm)

are made with a diamond knife and collected on Formvar-coated nickel grids (300 mesh). The area from which the ultrathin sections are made is selected from semithin 2 μm sections and counterstained with toluidine blue (1% (w/v) in distilled water, containing 2.5% (w/v) sodium carbonate; prior to use the staining solution is filtered through a 0.2 μm filter).

During ultramicrotomy, care should be taken that the front of the specimen block does not come into contact with the water bath of the diamond knife, so the water level must be set to a lower level than usually used for epoxy resin sections. In our experience, the Unicryl resin is relatively easy to section (few wrinkles) and the sections are stable in the electron beam.

2. PROTOCOLS FOR IMMUNOSTAINING

The protocols presented are used for all antigens, but dilution of the primary antibody has to be adapted.

2.1 Semithin sections

The procedure is applied on 2 μm sections of human liver, mounted on silanated glass slides (for the preparation of silanated glass slides, see Section 3). Before starting, it is necessary to make a scratch with a diamond point around the sections; otherwise it becomes impossible to localize the sections once the reagents are on the slide. Take care that the sections do not become dry during the procedure. Unless stated otherwise, all steps are done at room temperature. The glassware in which the silver enhancement solutions are mixed must be perfectly clean.

Procedure

(1) Sections are soaked in phosphate-buffered saline (PBS) (see note (a)); 5 min.
(2) Treat with Triton X-100 (1% in PBS); 5 min.
(3) Rinse in PBS; 5 min.
(4) Blocking of free aldehyde groups with 0.1 mol/L glycine in PBS; 15 min.
(5) Reduction of non-specific binding with 1% bovine serum albumin ((BSA) Fraction V; Sigma) in PBS; 60 min.
(6) Incubation with the antibody diluted in PBS (see note (b)); overnight at 4°C (in a humid chamber).
(7) Rinse with PBS; 3×10 min.
(8) Incubation with protein A–colloidal gold (5 nm) (note (c)) diluted in PBS (note (b)) containing 0.1% BSA (Fraction V; Sigma); 60 min.
(9) Rinse with PBS; 3×10 min.
(10) Stabilization (chemical fixation) of antigen–antibody–protein A–colloidal gold complex with 2.5% glutaraldehyde in PBS; 15 min.
(11) Rinse with PBS; 2×5 min.

(12) Rinse with double-distilled water; 3×5 min.
(13) Silver enhancement with IntenSE M (Amersham Life Sciences, code no. RPN 491) at room temperature for 4–6 min. An equal number of droplets from solutions A and B are mixed in a clean vial, immediately before use. Bring some droplets of the mixture onto the slides with a clean Pasteur pipette and ensure that all the sections are immersed.
(14) Rinse with double-distilled water; 3×2 min.
(15) Remove non-reduced silver with commercial photographic fixer solution (e.g. Hypam or Agefix diluted 1/20 in double-distilled water); 1 min. *According to the manufacturer's instructions this step is not required; however, in our experience additional silver precipitation may occur later in the non-fixed preparations. The photographic fixer is also applied to the cryostat and paraffin sections.*
(16) (i) Rinse with double-distilled water.

Optional (note (d))

(ii) Counterstain with toluidine blue (0.5% (w/v) solution in 0.01 mol/L HCl; pH 3.2) for 90 s at 45°C.
(iii) Rinse in two baths of tap water; 2×1 min.
(iv) Rinse with distilled water for 5 min.

(17) Dry the slides at 45°C (1 h) and mount (Fluoromount Mountant; Gurr Product No. 36098).

Notes: (**a**) Phosphate-buffered saline (PBS) is prepared as a 10× concentrated stock solution (80.0 g NaCl, 2.0 g KCl, 14.4 g $Na_2HPO_4.2H_2O$ and 2.0 g KH_2PO_4 in 1 litre distilled water). It can be stored at room temperature for several weeks. Prior to use, the solution is diluted 1/10 with distilled water. Check and adjust pH to 7.35. (**b**) The optimal dilutions have to be determined. (**c**) The protein A–colloidal gold is prepared by Dr J. W. Slot (Laboratory of Cell Biology, Utrecht, the Netherlands) according to the procedure described in Slot and Geuze (1985). Protein A–colloidal gold probes are commercially available. They remain stable over several months when stored at 4°C (do not freeze!). Always ensure that, when pipetting the required volume of the protein A–colloidal gold complex, the micropipette tip is clean. To prevent overall contamination, it is recommended to aliquot the stock. (**d**) Immunostained sections that have not been counterstained can be examined by dark-field illumination or phase-contrast microscopy to better visualize the tissue and/or the peroxisomes (Figures 3–6).

2.2 Ultrathin sections

The procedure, which is essentially the same as for the semithin sections, is applied on ultrathin (70–80 nm) sections collected on Formvar-coated nickel grids (300 mesh). Solutions of the different reagents are presented as droplets on a sheet of Parafilm. To prevent evaporation during the incubation with the primary antibody, a 'wet chamber' is used (the Parafilm lies on the bottom of a Petri dish containing a small vial filled with water). The grids float on top of the droplet; they are transferred using a loop of stainless steel wire. The back side of the grid should not become covered with any of the reagents and the side with the sections should not become dry during the procedure.

Procedure

(1) Soak with PBS; 2×5 min.
(2) Blocking of free aldehyde groups with 50 mmol/L NH_4Cl in PBS, 20 min, and with 100 mmol/L glycine in PBS, 20 min.
(3) Incubation with 2% BSA (Fraction V; Sigma) in PBS; 45 min.
(4) Primary antibody incubation; overnight at 4°C (in humid chamber).
(5) Rinse with PBS; 3×10 min.
(6) Incubation with protein A colloidal gold (15 nm) diluted in a 1:1 (v/v) mixture of PBS/5% (w/v) dry skim-milk powder in distilled water (adjust pH to 7.35); 45 min.
(7) Rinse with PBS; 3×10 min.
(8) Stabilization (chemical fixation) of antigen–antibody–protein A–colloidal gold complex with 2.5% glutardialdehyde in PBS; 15 min.
(9) Rinse with PBS; 5 min.
(10) Rinse with double-distilled water; 2×5 min.
(11) Conventional contrasting of the sections for electron microscopy with uranyl acetate (30 min) and lead citrate (5 min).

Negative controls

Incubation with primary antibody is replaced by incubation with normal rabbit serum or the IgG fraction of normal rabbit serum.

2.3 Results in control liver

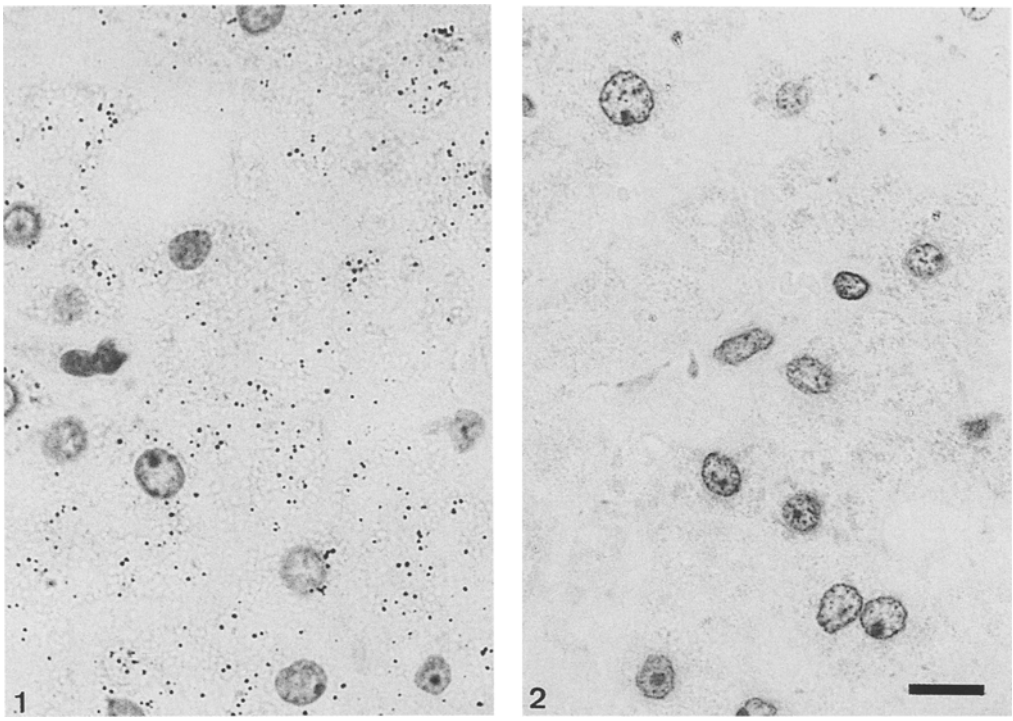
Semithin sections (Figures 1–6; 13)

Bright-field illumination: In human control liver, peroxisomes are visualized as small, brown-to-black granules, dispersed throughout the cytoplasm of the parenchymal cells after immunostaining against the peroxisomal matrix enzymes catalase, acyl-CoA oxidase, trifunctional enzyme, 3-ketoacyl-CoA thiolase and alanine:glyoxylate aminotransferase. Use built-in controls as a criterion to evaluate the specificity of the reaction and background level: (i) there are no immunoreactive granules over the sinusoids and over strands of connective tissue; (ii) inside the parenchymal cells, immunoreactive granules are absent over lipid droplets and the nucleus.

By fine focusing it can be seen that the gold–silver deposit is *only on the surface of the section* and not inside the section.

Phase-contrast illumination: The peroxisomes are visualized as blue granules against a grey background. The granules are better delineated than in bright-field microscopy.

Dark-ground illumination: The peroxisomes are seen as bright spots against a dark background. The spots are already detectable at low magnifications that do not permit one to see the organelles in the other illumination modes.

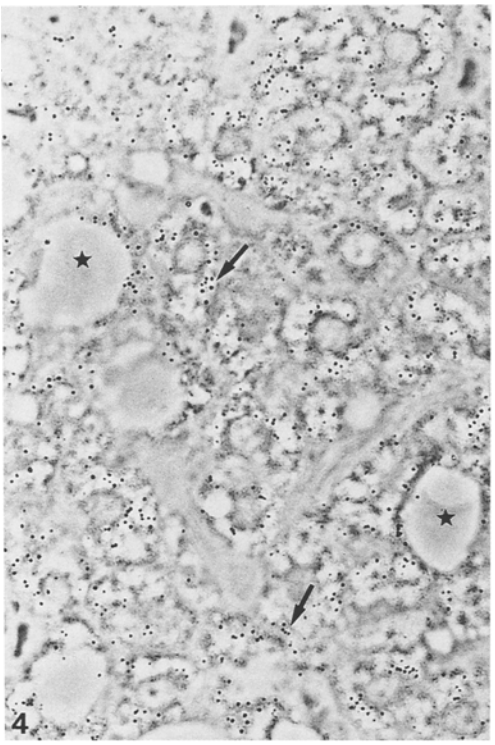


Figures 1 and 2 Semithin Unicryl section of human control liver, after immunostaining against catalase and counterstained with toluidine blue (**1**) and negative control incubation (**2**). The peroxisomes are visualized as small black granules throughout the parenchymal cells; the granules are absent in the negative control. Scale bar = 10 μm

Notes: (1) Especially in phase-contrast microscopy, granular structures can be seen not only at the surface of the section but throughout the whole section depth. These granules do not represent immunoreactive peroxisomes. They are also seen in the negative control incubations and in the sections counterstained with toluidine blue only. (2) Test the different illumination modes and compare their effect on peroxisome visualization. Dark-field illumination has an obvious enhancing effect on the visualization of the peroxisomes but concomitantly also of the background.

Ultrathin sections

In control liver, the colloidal gold particles are concentrated over the peroxisomal profiles after labelling for the above antigens (Figures 7–11). There is no significant label over any other subcellular structure. The negative control incubations do not show a labelling pattern; a few randomly dispersed gold particles are usually present throughout the section (Figures 12 and 20).



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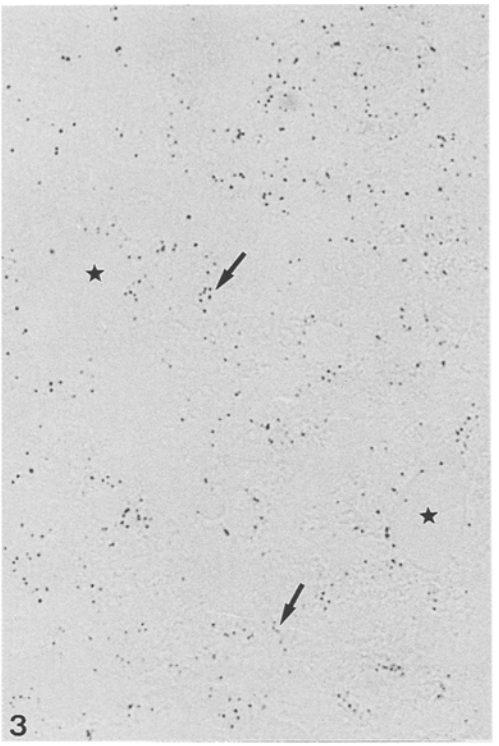

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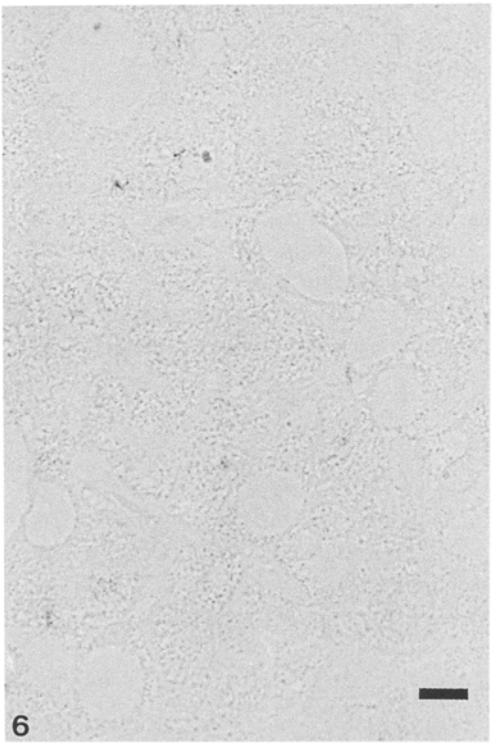

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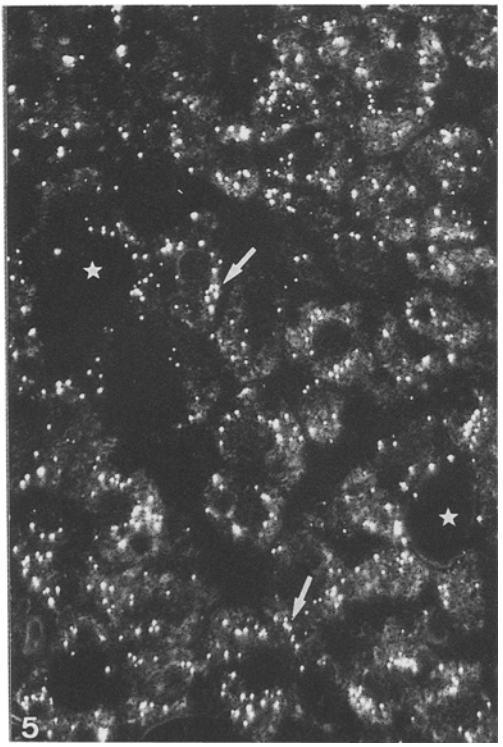

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Remark: The ultrastructural detail in ultrathin acrylic resin sections differs markedly from that in conventional epoxy resin sections. The overall image has a lower contrast and the membranes of the organelles are poorly visualized, providing a less 'aesthetic' picture (term of Griffiths 1993); the mitochondrial cristae seem negatively stained after uranium and lead. In the liver, an additional characteristic is that the glycogen rosettes are not contrasted at all, leading to the image of an empty cytoplasm.

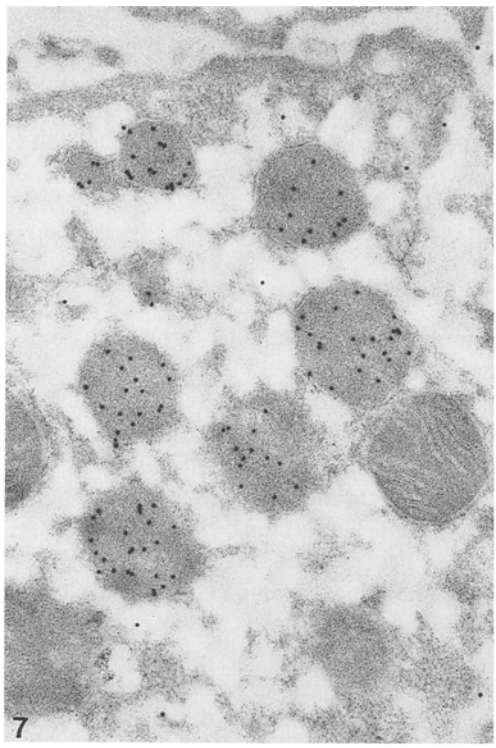
2.4 Comments and methodological problems

The use of semithin acrylic resin sections for immunodetection

Advantages: (1) Except for the silver enhancement on the semithin sections and the different diameter of the colloidal gold particles (5 μm versus 15 μm), the immunodetection method is similar for the semithin and ultrathin sections. As a result, the light-microscopic and ultrastructural images can be correlated at a detailed level. This has proved to be an advantage for the interpretation of some peculiar images: in some patients enlarged peroxisomes containing fat droplets and elaborate membrane invaginations are present; these inclusions, which are devoid of peroxisomal matrix, can be seen at the light-microscope level as a pale spot inside the organelle (compare Figures 14 and 15). Secondly, it is possible to first perform labelling on semithin sections and then to select an area for ultramicrotomy and proceed further on ultrathin sections.

(2) The labelling procedure does not require etching of the resin or trypsinization of the tissue, the latter being a critical step in unmasking the antigen in paraffin sections of formaldehyde-fixed liver (Litwin et al 1988). It seems that in the semithin acrylic resin sections the antigen is exposed — at the surface of the section — in a directly accessible and recognizable way for the antibody, without any pretreatment. This is an important advantage: on paraffin and cryostat sections of several control liver biopsies, peroxisomes were visualized only in the outer margin of the section, after immunostaining against catalase and the peroxisomal β-oxidation enzymes. This margin was sometimes only a few cells wide and the internal part of the section was unreactive. From diaminobenzidine cytochemistry in the same tissue block, it was clear that the peroxisomes were uniformly present in all the parenchymal cells. Litwin and co-workers have encountered similar problems in their formaldehyde-fixed samples, which they could resolve by adequate trypsinization (Jan Litwin, personal communication). In our experience, trypsinization did not show a beneficial effect in *all* the samples. Therefore, the phenomenon might lead to false negative results in the paraffin and cryostat sections in the case of a peroxisomal localization of the antigen. In the semithin and ultrathin sections the 'margin effect' was never observed. When the antigen is present in the *cytoplasm* (e.g. cytoplasmic catalase in

Figures 3–6 Semithin Unicryl section of human control liver (alcoholic liver steatosis) after immunostaining against alanine:glyoxylate aminotransferase without counterstaining, viewed in bright-field microscopy (**3**), phase-contrast microscopy (**4**), and dark-field microscopy (**5**). The asterisks indicate large fat droplets; the arrows indicate groups of peroxisomes for reference in the different illumination modes. There are no granules over the lipid droplets. The negative control incubation viewed in bright-field is presented in **Figure 6**; immunoreactive granules are absent. Scale bar = 10 μm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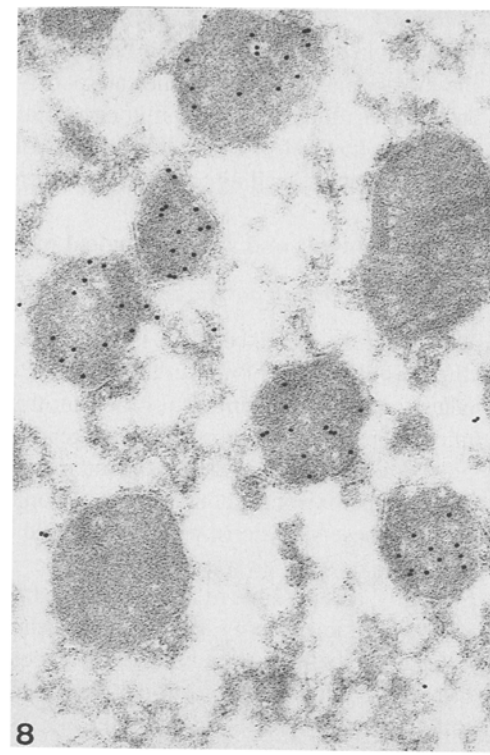
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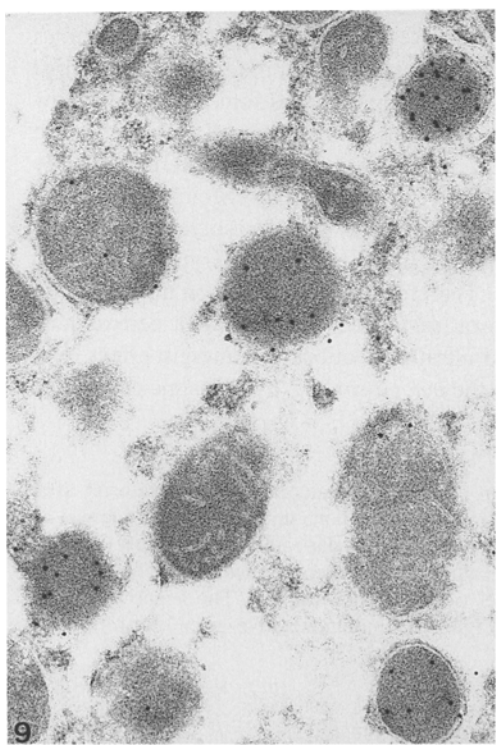
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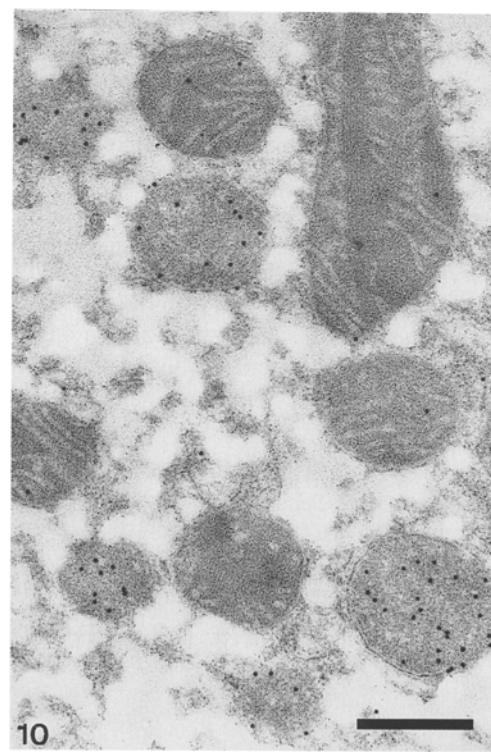
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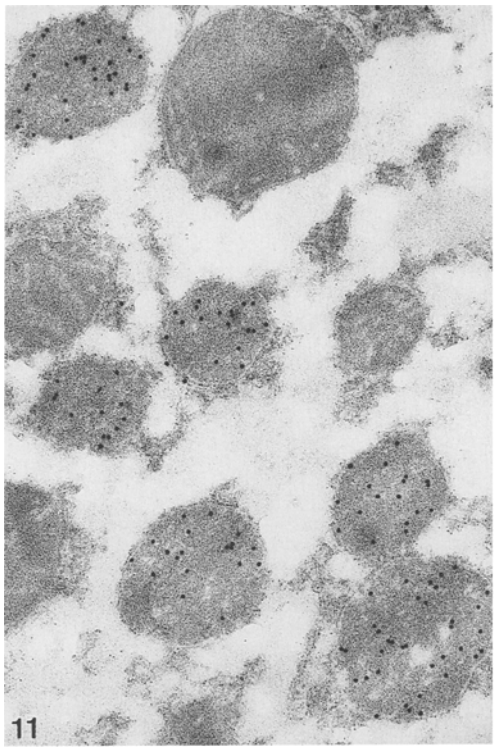
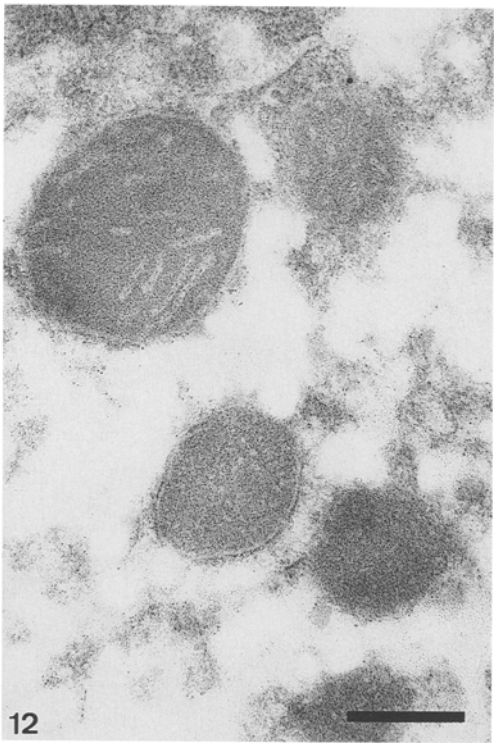




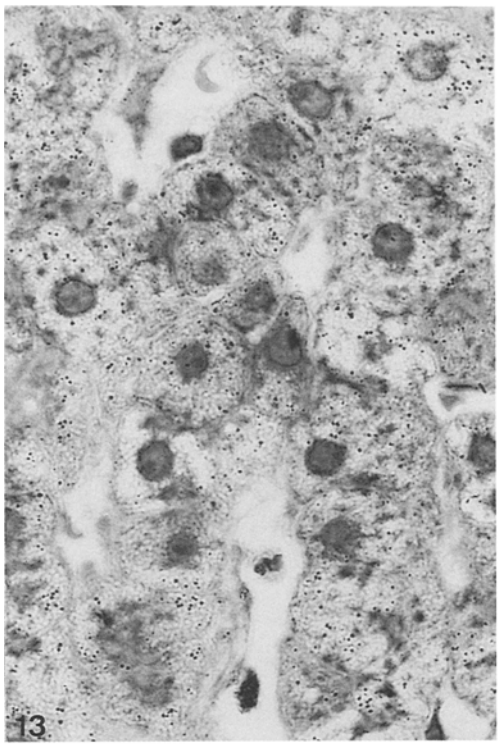
Figures 7–12 Immunodetection of catalase (**7**), acyl-CoA oxidase (**8**), trifunctional enzyme (**9**), 3-ketoacyl-CoA thiolase (**10**), and alanine:glyoxylate aminotransferase (**11**) in ultrathin Unicryl sections of human control liver, and a negative control incubation (**12**). In Figures 7–11 the peroxisomal profiles are labelled with gold particles; there is no distinct label in the negative control incubation (see also Fig. 17). Scale bar=0.5 μm

the generalized peroxisomal disorder patients), the margin effect does not occur in the cryostat sections stained according to the previously published protocol (Espeel et al 1990a). Also in formalin-fixed archival samples and in autopsy samples, the margin effect was not observed.

(3) Since only peroxisomes at the section surface are detected, the number of visualized organelles does not depend on section thickness.

(4) In tissues embedded in acrylic resins, the antigenicity of many proteins is preserved over long periods; the resin behaves as a relatively inert medium. As a result, the embedded material can be examined retrospectively in view of new questions or with newly available antibodies. By embedding a control liver biopsy at regular intervals, closely matching positive control tissue is present throughout the sample collection.

(5) The mounted colloidal gold–silver immunostained sections (acrylic resin, paraffin and cryostat sections) remain stable over several years; there is no apparent loss or diffusion of the signal or of the tissue structure.



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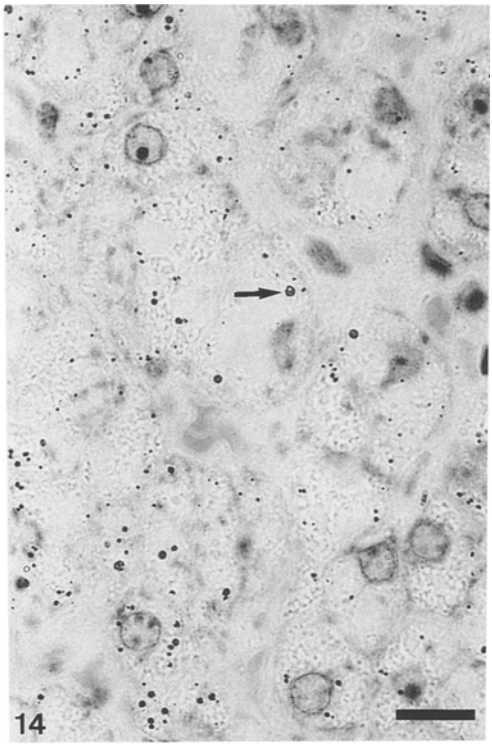

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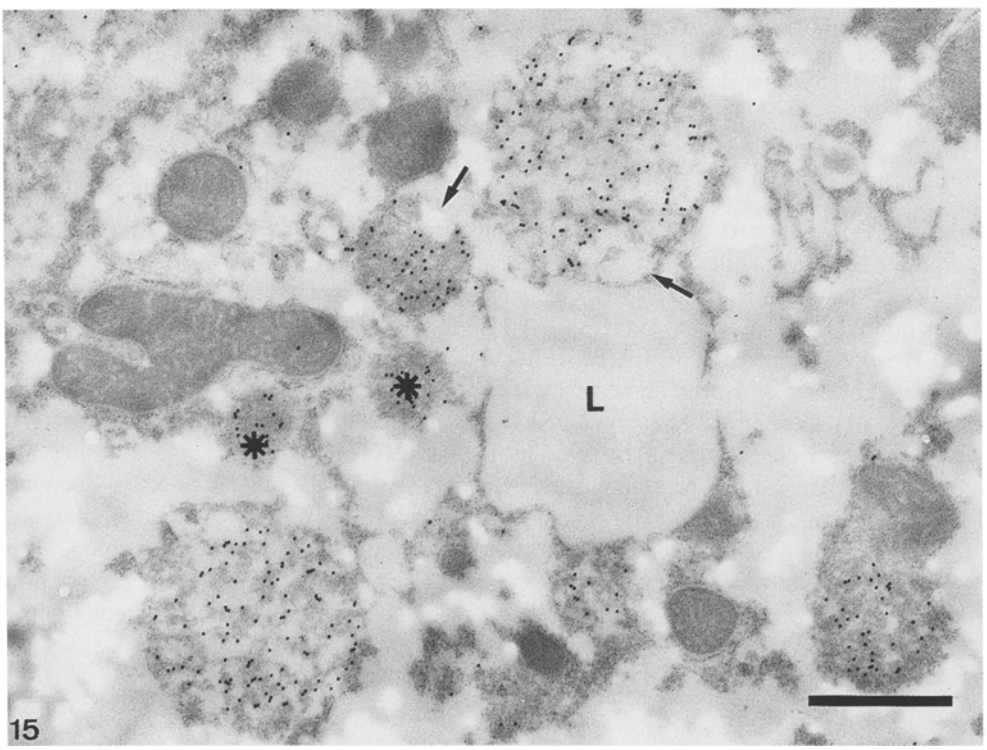

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Disadvantages: (1) In the semithin 2 μm sections a weak to negative signal is obtained when an antigen is dispersed in the cytoplasm (e.g. catalase in patients with a generalized peroxisomal disorder). This is most probably related to the fact that the layer of colloidal gold particles (which are only on the section surface) is not dense enough for the silver to form a uniform deposit. In cryostat sections stained as described previously (Espeel et al 1990a), and in the ultrathin Unicryl sections, the cytoplasmic localization of catalase can be clearly demonstrated (Figures 16–20; Espeel et al 1993; Roels et al 1993); for its demonstration by staining for catalase activity see Roels et al (1995b).

(2) Because of the small size of the embedded tissue fragments, relatively small areas of tissue can be examined in one section. Several specimen blocks have to be sectioned.

(3) A silver/gold deposition on the resin, often just outside the tissue, may be seen occasionally. It does not occur in a consistent way: on the same slide it may be present on only some sections.

(4) The period between sampling the biopsy and the first immunostained semithin sections is about 2 weeks.

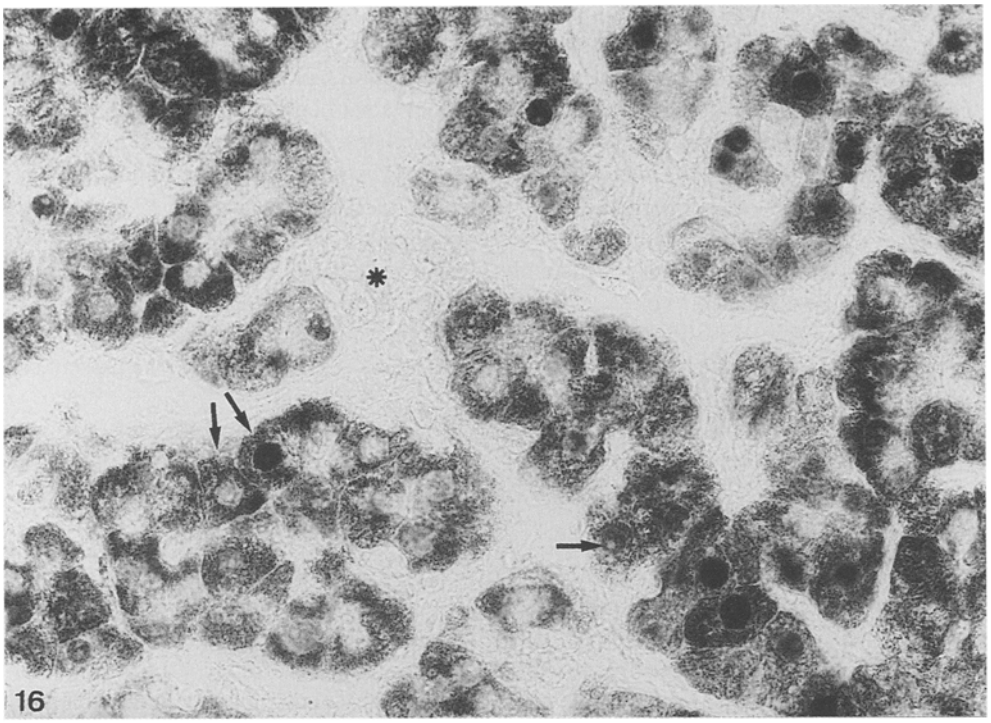
Limitations

The immunocytochemical detection relies on *the presence of the enzyme protein and not on its activity.* Even a small part of the molecule that still contains an epitope can give an immunopositive signal. Therefore, only the absence of a peroxisomal protein — as detected by immunocytochemistry — can be of diagnostic value. So far, we have found a normal peroxisomal localization of the three peroxisomal β-oxidation enzyme proteins in liver biopsies from three patients suffering from a peroxisomal β-oxidation defect who were clinically different from X-linked adrenoleukodystrophy (one case has been reported previously; in the kidney a peroxisomal localization of the three β-oxidation enzymes was also found (Espeel et al 1991a; Van Maldergem et al 1992)). Fibroblast complementation studies by Wanders et al (1992) revealed that in this patient the impaired peroxisomal β-oxidation was due to the inactivity of bi(tri)functional enzyme (R. J. A. Wanders, personal communication).

On the other hand, we could not detect immunoreactive enzyme protein in the peroxisomes of three cases: alanine:glyoxylate aminotransferase in two patients with primary hyperoxaluria type I, and acyl-CoA oxidase in a fetus from an interrupted pregnancy in the family described by Poll-The et al (1988). In a variant form of

Figures 13 and 14 Peroxisomes after immunolabelling for alanine:glyoxylate aminotransferase in semithin sections of a control liver (**13**) and of a chondrodysplasia punctata variant (**Fig. 14**; patient 4 in table 1 of Kerckaert et al 1995; biopsy at 11 years). In the patient's biopsy the peroxisomes are less numerous and many organelles are markedly enlarged. The arrow indicates an empty spot inside an enlarged peroxisome. Compare with Fig. 15 for the result at the ultrastructural level. Scale bar = 10 μm

Figure 15 Immunolabelling for alanine:glyoxylate aminotransferase in ultrathin sections of the specimen presented at the light-microscopic level in Fig. 14. The heterogenous size of the organelles, their reticular matrix and the empty places inside the matrix (arrows) are illustrated. Two normal-appearing organelles (asterisk) are also illustrated. L = lipid droplet. Scale bar = 1 μm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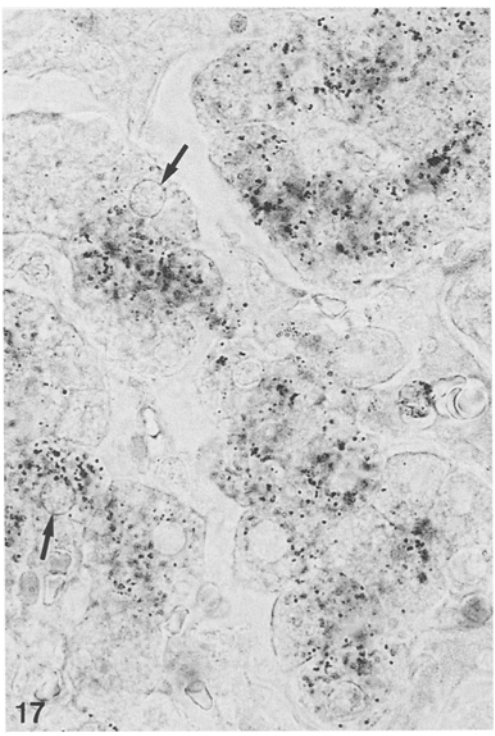
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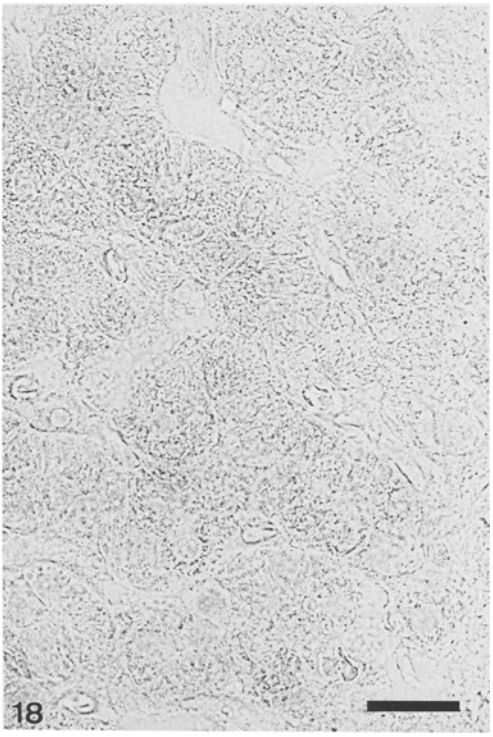
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chondrodysplasia punctata described by Smeitink et al (1992) rare and extremely enlarged peroxisomes that were largely catalase-deficient were present in the liver biopsy. The organelles contained the three peroxisomal β-oxidation enzymes as well as alanine:glyoxylate aminotransferase; catalase was mainly localized in the cytoplasm and no normal catalase-containing peroxisomes were found (Espeel et al 1993). In two 'classical' rhizomelic chondrodysplasia punctata patients, Hughes et al (1992) also reported extremely enlarged and catalase-deficient peroxisomes in the liver.

In addition, no data are obtained by immunocytochemistry about the molecular processing of the reactive protein: for example, the unprocessed form of 3-ketoacyl-CoA thiolase (44kDa) and the mature form (41 kDa) cannot be discriminated by our polyclonal antibodies.

Visualization of peroxisomes in autopsy and archival samples

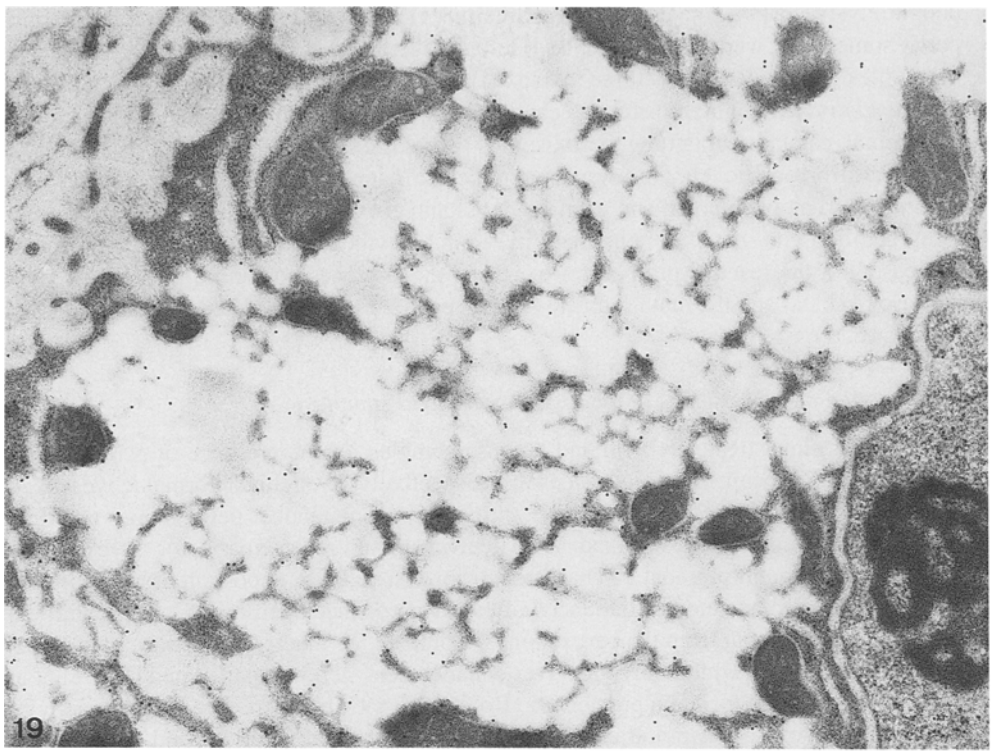
It is useful to investigate archival and autopsy samples for the presence of peroxisomes, e.g. in order to document an index case retrospectively. In archival formaldehyde-fixed liver, immunocytochemistry for catalase antigen is recommended: peroxisomes could be identified in liver samples that had been kept in unbuffered formalin for 5 years. The presence of 'formalin-pigment', a characteristic brown-to-black deposit in tissues stored in formalin that becomes more pronounced after silver enhancement, may interfere with the immunopositive signal from the peroxisomes. Compare the image with unstained sections and with sections on which only the silver enhancement step was performed (Espeel et al 1991b; for images of formalin pigment see Roels et al, 1995b). Also, the immunoreactivity for alanine:glyoxylate aminotransferase may be preserved over several years (own unpublished observations).

In human autopsy liver, the three peroxisomal β-oxidation enzymes, in addition to catalase, are detectable up to at least 55 hours after death (Espeel et al 1990a). By immunolabelling against catalase and 3-ketoacyl-CoA thiolase, the hepatic peroxisomes could be visualized in a stillborn fetus at 26 weeks of gestation affected with X-linked recessive chondrodysplasia punctata (Van Maldergem et al 1991).

Human kidney

We have immunostained paraffin and cryostat sections of human kidney samples for catalase and the peroxisomal β-oxidation enzymes according to the protocol described previously (Espeel et al 1990a). On paraffin sections from a patient with a generalized

Figures 16–18 Immunolabelling for catalase (**16**) in 8 μm cryostat liver section of a generalized peroxisomal disorder patient. Catalase staining reveals a diffuse immunoreactivity in the cytoplasm of the parenchymal cells. The staining intensity varies between individual cells; there is no reaction in non-parenchymal tissue (asterisk). A positive reaction is also seen in a part of the nuclei; the reactivity differs between nuclei of adjacent cells (large arrows). The nucleolus is unreactive (small arrow). **Figure 17** shows catalase immunoreactivity in the peroxisomes (visualized as dark granules) of a control liver (cryostat section); there is no evidence for a reaction in the cytoplasm or in the nuclei (arrow). **Figure 18** shows the negative control incubation of the generalized peroxisomal disorder patient. Scale bar = 20 μm. The procedure for cryostat sections is described in Espeel et al (1990a); in addition, photographic fixer was applied (see protocol in Section 2.1, step 15)



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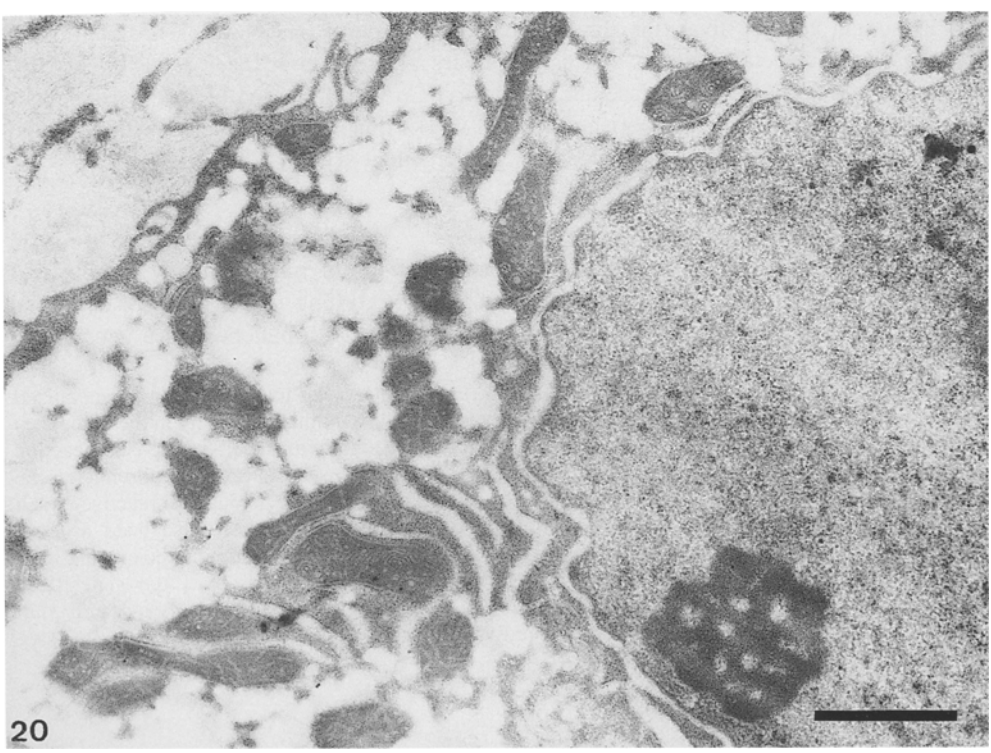

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peroxisomal disorder, a distinct cytoplasmic localization of catalase together with a reaction in some nuclei was found in the epithelial cells of the proximal tubules (Figures 21 and 22). We found a normal peroxisomal localization of the three peroxisomal β-oxidation enzymes in the proximal tubules of the neonate with the peroxisomal β-oxidation defect (Espeel et al 1991a).

Also, in archival formalin-fixed kidney samples, the peroxisomes could be identified via immunostaining for catalase in cryostat and paraffin sections (unpublished observations).

Setup for biopsies from a patient with a suspected peroxisomal disorder

Each incubation experiment on semithin and ultrathin sections from a patient with a suspected peroxisomal disorder involves a simultaneous incubation of at least one control liver. For each analysis, we treat 10–12 slides/grids at most: primary antibodies for two antigens in two different dilutions and a negative control incubation in both patient and control samples. In case of an aberrant localization pattern or reaction, the procedures are repeated in combination with a parallel treatment of a human control sample. Other cytochemical stains are described by Roels et al (1995b).

3. PREPARATION OF SILANATED GLASS SLIDES

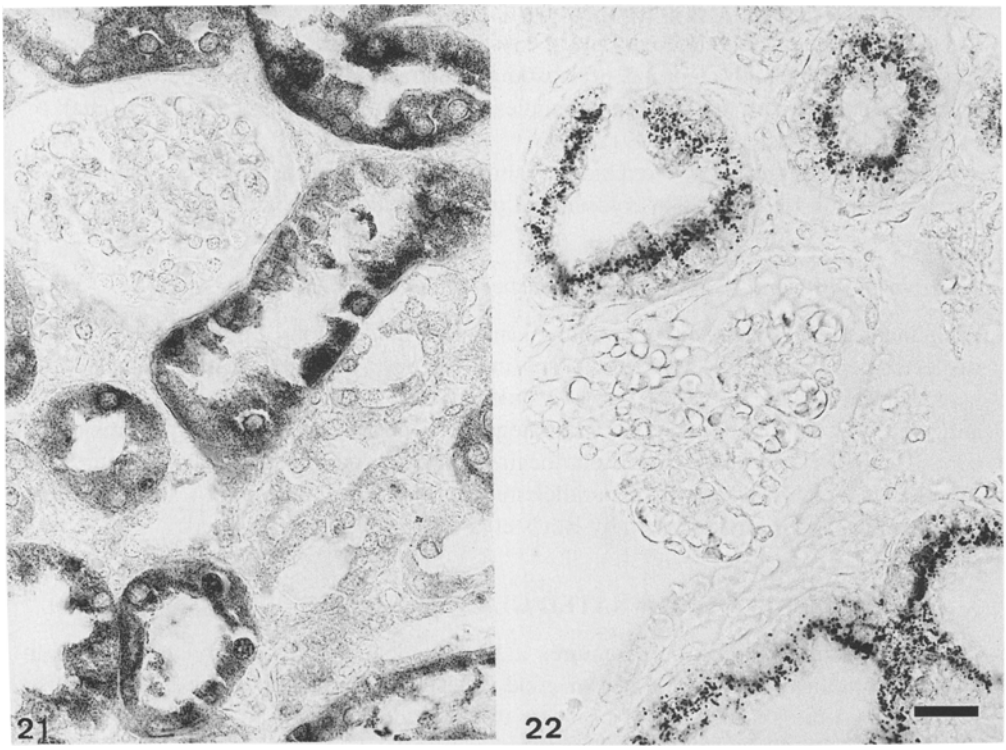
The immunostaining procedure requires a long incubation of the acrylate sections in aqueous solutions. Without the use of good adhesives, this results in the detachment of sections from the slides. Several products that provide maximal adhesion and minimal interference with the immunoreagents are commercially available.

A very efficient adhesion method that produces a low background is coating of the glass slides with organosilanes according to Henderson (1989). The binding mechanism is not known exactly; it is assumed that the organosilanes act as linker reagent (either ionically or covalently) between aldehyde, amino and ketone groups in the tissue and hydroxyl groups at the glass surface. There is no apparent reaction with silver solutions, cationic and anionic dyes, fat-soluble dyes, Schiff reagent or antibodies. Sections on silane-coated slides do not detach after digestion with amylase, trypsin, pronase, pepsin, protease or microwave staining. Coating with aminoalkylsilanes provides superior tissue adhesion — except for tissues fixed with mercury-containing solutions — and less background reaction than with slides coated with albumen, chrome-gelatin or poly-L-lysine (Henderson 1989).

Procedure

(1) Clean glass slides are dipped in a solution of 2% 3-aminopropyltriethoxysilane (APS; A-3648 Sigma, Munich) in acetone for 5 min at room temperature.

Figures 19 and 20 Immunolocalization of catalase in a patient with a generalized peroxisomal disorder; ultrathin Unicryl section (**19**). Labelled peroxisomes are absent. The gold particles are distributed over the cytoplasm and nucleoplasm but not the nucleolus; the perinuclear cisterna, mitochondrial profiles, sinusoidal lumen and space of Disse are relatively devoid of gold particles, similar to the negative control incubation (**20**). Scale bar = 1 μm



Figures 21 and 22 Immunostaining for catalase in the kidney cortex of a patient with a generalized peroxisomal disorder, paraffin section (**21**). A diffuse staining in the cytoplasm and some nuclei of the epithelial cells in the proximal tubules is observed; there is no punctate staining pattern reflecting a peroxisomal localization of catalase as in the control kidney (**22**; cryostat section). Scale bar = 20 μm

(2) The slides are immersed in deionized water for 2×5 min.
(3) Leave the slides to air dry or dry at 40°C for 2 days.
(4) When completely dry, the slides are stored dust-free at room temperature.

Four sections are placed in a droplet of distilled water on the glass slide. The slides are then left to dry at 40°C for at least 2 days prior to immunostaining.

Remark: The adhesive properties are not lost from slides stored for 6 months, or for sections picked up and stored for 6 months (Henderson 1989).

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