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Epoxide hydrolase is a marker for the smooth endoplasmic reticulum in rat liver

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Epoxide hydrolase (EH, EC 3.3.2.3) was chosen as a potential marker for smooth endoplasmic reticulum, because this enzyme is inducible by drugs such as phenobarbital. The hypothesis was verified in rat liver using immunochemical and immunocytochemical techniques. Antibodies were raised to the purified protein. These antibodies were affinity purified using the enzyme immobilized on Sepharose Ultrogel. The specificity of the antibodies was assayed by immunoelectrotransfer (Western blot). The labelling of rat liver thin frozen sections with protein A-gold particles demonstrated that the antibodies specifically recognised smooth endoplasmic reticulum membranes. Rough endoplasmic reticulum, other intracellular organelles and plasma membrane were unlabelled.
Key words: epoxide hydrolase/endoplasmic reticulum/antibodies

Introduction

Over the past few years attempts have been made to discover the molecular basis of the individuality of intracellular organelles or membranes. One of the most promising approaches is to obtain antibodies which recognise an antigen located exclusively within one organelle or a specific domain of one organelle (Brown and Farquhar, 1984). Different methods have been developed to obtain such antibodies, either using purified antigens of known origin (Meyer *et al.*, 1982a; Roth and Berger, 1982; Hiller and Weber, 1982; Louvard *et al.*, 1983) or using monoclonal antibodies (Burke *et al.*, 1982; Louvard *et al.*, 1983) or by immunising animals with the purified membrane of the desired organelle and then absorbing non-specific antibodies from the antiserum obtained. Using this last procedure, antibodies have been raised specific to the rough endoplasmic reticulum (RER), to the Golgi complex (Louvard *et al.*, 1982) and the lysosome membranes (Reggio *et al.*, 1984). These antibodies have been very useful for following molecular events associated with the vectorial transport of newly synthesized proteins through the membranes of the RER (Meyer *et al.*, 1982b), the transport of viral proteins to the plasma membrane (Green *et al.*, 1981), membrane reorganisation during mitosis (Burke *et al.*, 1982; Schroeder *et al.*, 1984) and the rearrangement of the microtubule organisation center in migrating cells (Kupfer *et al.*, 1982, 1983).

We wished to obtain antibodies which would be specific for the smooth endoplasmic reticulum (SER). Since it is not yet possible to isolate pure samples of SER membrane we looked for an enzyme which might be present only in this organelle and which would be easy to purify. A good candidate is epoxide hydrolase (EH, EC 3.3.2.3) since this enzyme activity is increased about seven times in liver by a 5-day treatment with phenobarbital (Oesch, 1973). This treatment is known to double the SER membrane area when the RER membrane area does not change (Stäubli *et al.*, 1969; Remmer and Merker, 1965; Jones and Fawcett, 1966). Several other enzymatic activities involved in drug metabolism (Vainio, 1973; Magdalou *et al.*, 1982) are also increased. Moreover, epoxide hydrolase has been well characterised (for a recent review, see Oesch, 1983). Here we show by cell fractionation and immunocytochemistry that the membrane-bound epoxide hydrolase can be used in rat liver as a marker for the smooth endoplasmic reticulum.

Results

Characterisation of the antibodies to epoxide hydrolase

Antibodies were raised to EH (isolated from microsomal membranes) (mEH_b) and affinity purified on the pure enzyme, immobilized on Ultrogel beads. The purified antibodies were tested by immunoelectrotransfer on the pure enzyme, on a total microsomal fraction and on the whole homogenate (Figure 1). On the purified enzyme the antibodies recognised a major band with an apparent mol. wt. of 50 kd which is the enzyme's mol. wt. (Lu *et al.*, 1979; Heinemann and Ozols, 1984) (Figure 1b). Two additional bands (mol. wt. 116 and 200 kd) were also faintly

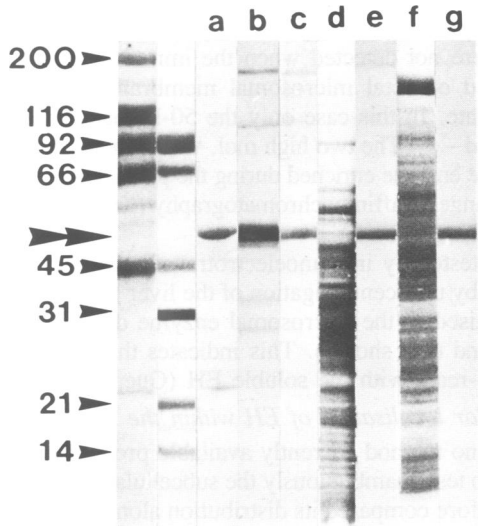

Fig. 1. Characterization of the antibodies raised to liver epoxide hydrolase (mEH_b). The pure enzyme **(a)**, the crude microsomal liver fraction **(d)**, the whole liver homogenate **(f)** and the whole BHK cell homogenate **(h)** were separated on SDS-PAGE and stained with Coomassie blue. Similar strips were transferred on nitrocellulose and labelled with affinity-purified antibodies against EH followed by sheep anti-rabbit IgG conjugated with horseradish peroxidase (respectively **b, e, g** and **i**). In **lane c**, the labelling was performed as in **b** with the antibodies affinity purified on the nitrocellulose strip containing the 50-kd band. Mol. wt. markers: myosin (200 kd), β-galactosidase (116 kd), phosphorylase B (92 kd), BSA (66 kd), ovalbumin (45 kd), carbonic anhydrase (31 kd), soybean trypsin inhibitor (21 kd) and lysozyme (14 kd).

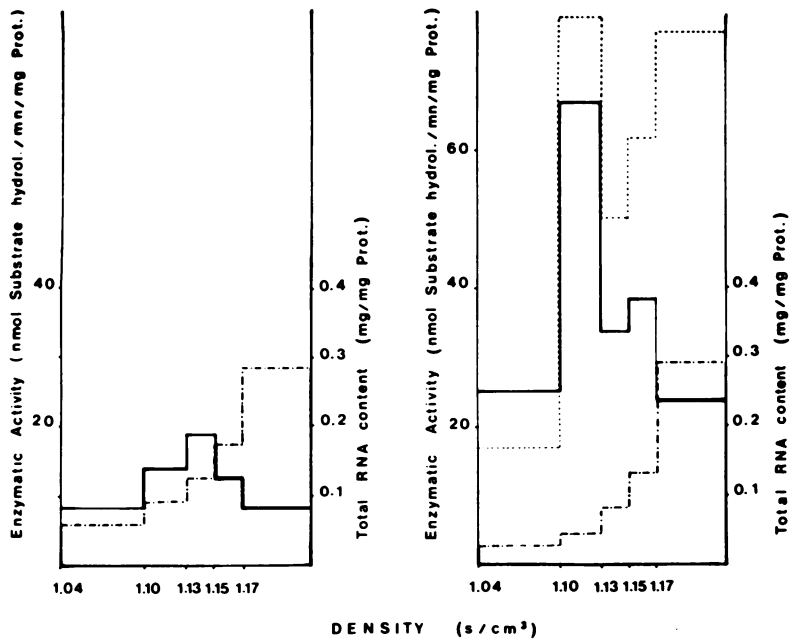

Fig. 2. Distribution of EH (-·-·-) and UDPGT (·····) activities and total RNA content (—) after subfractionation of total microsomal membranes on a step sucrose gradient. **(a)** Control liver, **(b)** phenobarbital-treated liver. 25% of the total homogenate activity was loaded on the gradient.

recognised. These bands were not visible on the Coomassie blue staining of the corresponding SDS-PAGE (Figure 1a). Purified EH was further analysed on SDS-PAGE and transferred onto nitrocellulose sheets, the 50-kd band was cut and the antibodies were affinity purified on the nitrocellulose strip according to Olmsted (1981). The resulting antibodies still recognised the three bands, indicating that the 116-kd and 200-kd bands are immunologically related to the 50-kd enzyme (Figure 1c). These two bands were not detected when the immunoelectrotransfer was performed on total microsomal membranes or on the whole homogenate. In this case only the 50-kd band was recognised (Figure 1d—g). The two high mol. wt. antigens could be precursors of the enzyme enriched during the purification resulting from ion exchange or affinity chromatography (Knowles and Burchell, 1977).

When tested by immunoelectrotransfer on a soluble fraction obtained by ultracentrifugation of the liver homogenate the antibodies raised to the microsomal enzyme did not recognise the 50-kd band (not shown). This indicates that our antibodies do not cross-react with the soluble EH (Guenthner *et al.*, 1981).

Subcellular localisation of EH within the SER fractions

There is no method currently available providing an SER pure enough to test unambiguously the subcellular localisation of EH. We therefore compared its distribution along a sucrose step gradient. Total endoplasmic reticulum (ER) preparations from control and phenobarbital-treated rat livers were subfractionated for 3 h at 100 000 *g*, conditions in which the particles approach their equilibrium position (Beaufay *et al.*, 1974). Each fraction was analysed for EH activity (mEH_b), total RNA content (as a marker for RER) and for UDP glucuronosyltransferase (UDPGT) activity, an enzyme widely distributed in the rough and the smooth ER membranes when the substrate used is 4-methylumbelliferone (Beaufay *et al.*, 1974) (Figure 2); differential distribution has been found with other substrates (Stasiecki *et al.*, 1980). Epoxide hydrolase activity was found in the lightest parts of the gradient where the RNA content was the lowest. These fractions were mainly composed of SER vesicles known to equilibrate at a density between 1.05 and 1.18 (Gram, 1974). RNA was never totally absent from any fraction due to contamination of the SER fractions by some RER vesicles easily recognisable by electron microscopy (not shown) although we chose the limit of 1.17 for density to minimise this contamination. Similarly, 10.1% of the normal and 15.6% of the phenobarbital-treated liver EH activities were found in the heavy fractions of the gradients where the RER vesicles equilibrate ($d > 1.17$) and where the RNA concentration was higher (Beaufay *et al.*, 1974). UDPGT activity was distributed among all fractions of the gradient. Phenobarbital treatment promoted a better separation of enzymatic activities along the gradient. The SER vesicles equilibrated at a density slightly lighter and the RNA-containing vesicles were more densely packed at the bottom of the gradient.

Antibodies to EH-labelled SER membranes by immunocytochemistry

The labelling of the SER by antibodies to EH was tested using frozen sections of normal and phenobarbital-treated rat liver. At the light microscope level the labelling was restricted to specific zones scattered throughout the cell cytoplasm (Figure 3a and c). The plasma membrane and the nuclei were devoid of labelling. No significant labelling was observed over the endothelial cells (which have very little SER), over the erythrocytes and the capillary lumen. Treatment with phenobarbital did not change the pattern observed but the area and the intensity of the labelling within hepatocytes were significantly increased. This pattern was totally different from that obtained by using antibodies to the RER (Figure 3d).

At the electron microscope level the labelling was associated with the SER membranes (Figures 4, 5 and 6). These membranes were localised in specific areas distinct from those containing other cellular organelles (RER, mitochondrion, etc.) (Figures 4 and 5). This can explain the observations made with the light microscope. The nuclei, the mitochondria, the peroxisomes, the Golgi complex including the coated vesicles and the plasma membranes were not labelled (Figures 4, 5 and 6). The labelling of the membranes of the RER was not significantly higher than the

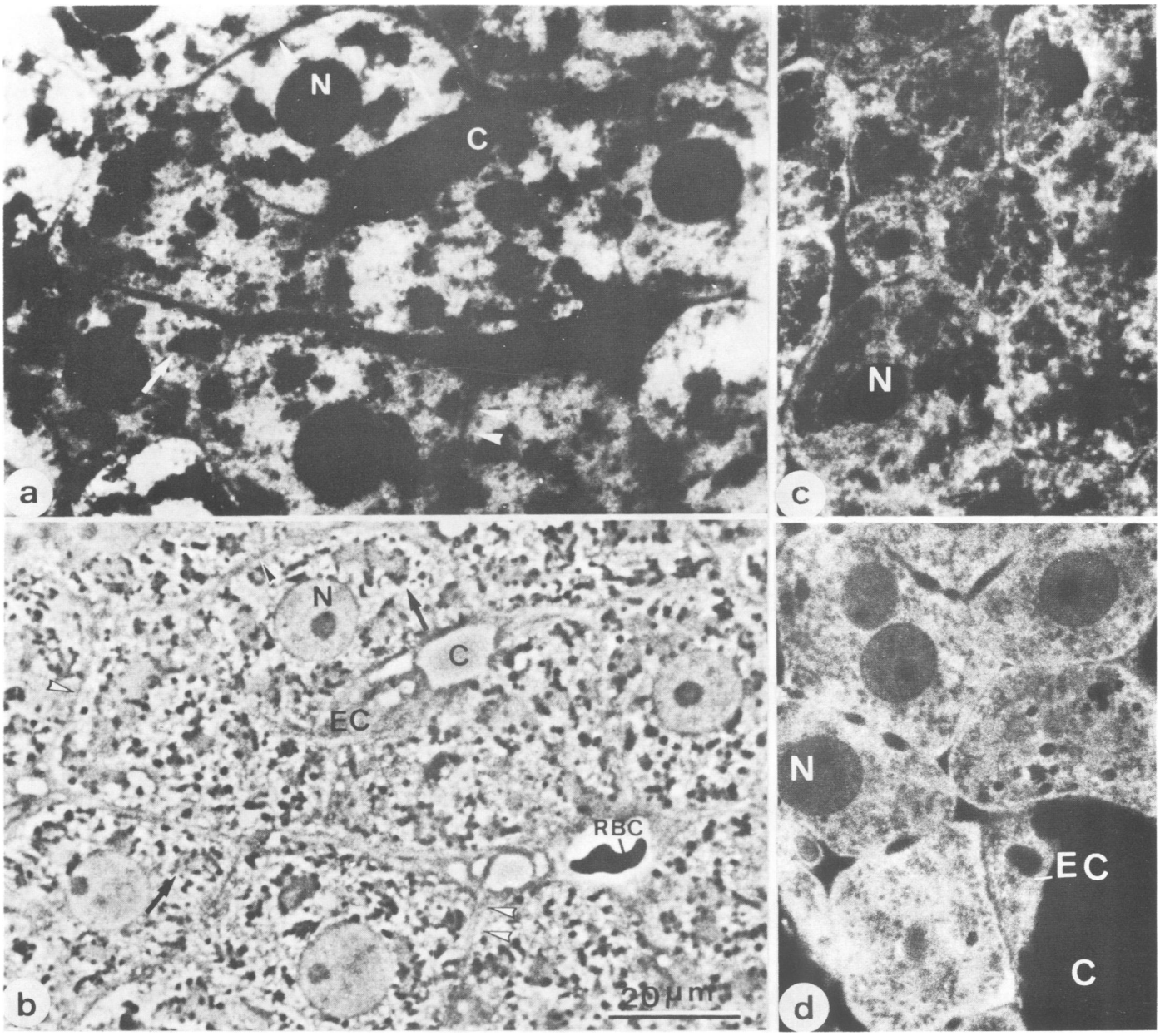

Fig. 3. Frozen thin sections from rat liver stained for immunofluorescence with antibodies to EH **(a)** tissue induced by phenobarbital. The labelling was restricted to very specific areas of the cell cytoplasm whereas other areas were completely devoid of labelling (arrows). The plasma membranes (arrowheads) and the nuclei (N) were not labelled. The endothelial cells (EC), the red blood cells (RBC) and the capillary lumen (C) were not labelled. **(b)** Is the phase contrast optics of the same field. Although the general features of the liver are similar to the induced liver the area and the intensity of the labelling was weaker than **(a)**. **(c)** Is a control liver tissue. **(d)** Shows the labelling of induced liver with antibodies to RER. × 1000; bar = 20 µm.

background, observed over the membranes of other cytoplasmic organelles.

Discussion

By a combination of biochemical and immunocytochemical methods we have demonstrated that EH is localised mainly within the SER membranes and can be used as a marker for this organelle.

ER proteins can be divided into three different classes. Most of them are widely distributed in both the rough and the smooth membranes as shown by differential centrifugation (Beaufay *et al.*, 1974; Amar-Costesec *et al.*, 1974) and immunolabelling (Fowler *et al.*, 1976; Remacle *et al.*, 1976). More recently it was demonstrated that some ER proteins such as the ribophorins (Kreibich *et al.*, 1978) or the docking protein (Meyer *et al.*, 1982b) were restricted to the rough membranes. Here we report a new class of proteins that is mainly restricted to the smooth membranes of the endoplasmic reticulum. Although membrane markers are never absolute (Howell *et al.*, 1978) the two last classes can be used to define the two ER compartments on a molecular basis.

Using fractionation by differential centrifugation different authors found EH activity both in rough and smooth endoplasmic reticulum membranes. The activity was highest, however, in SER fractions (Stasiecki *et al.*, 1980; Bentley *et al.*, 1980; Vogel-Bindel *et al.*, 1982). We observed the same type of results by similar methods but using a more refined fractionation, i.e., a sucrose step gradient, we found that EH activity was associated

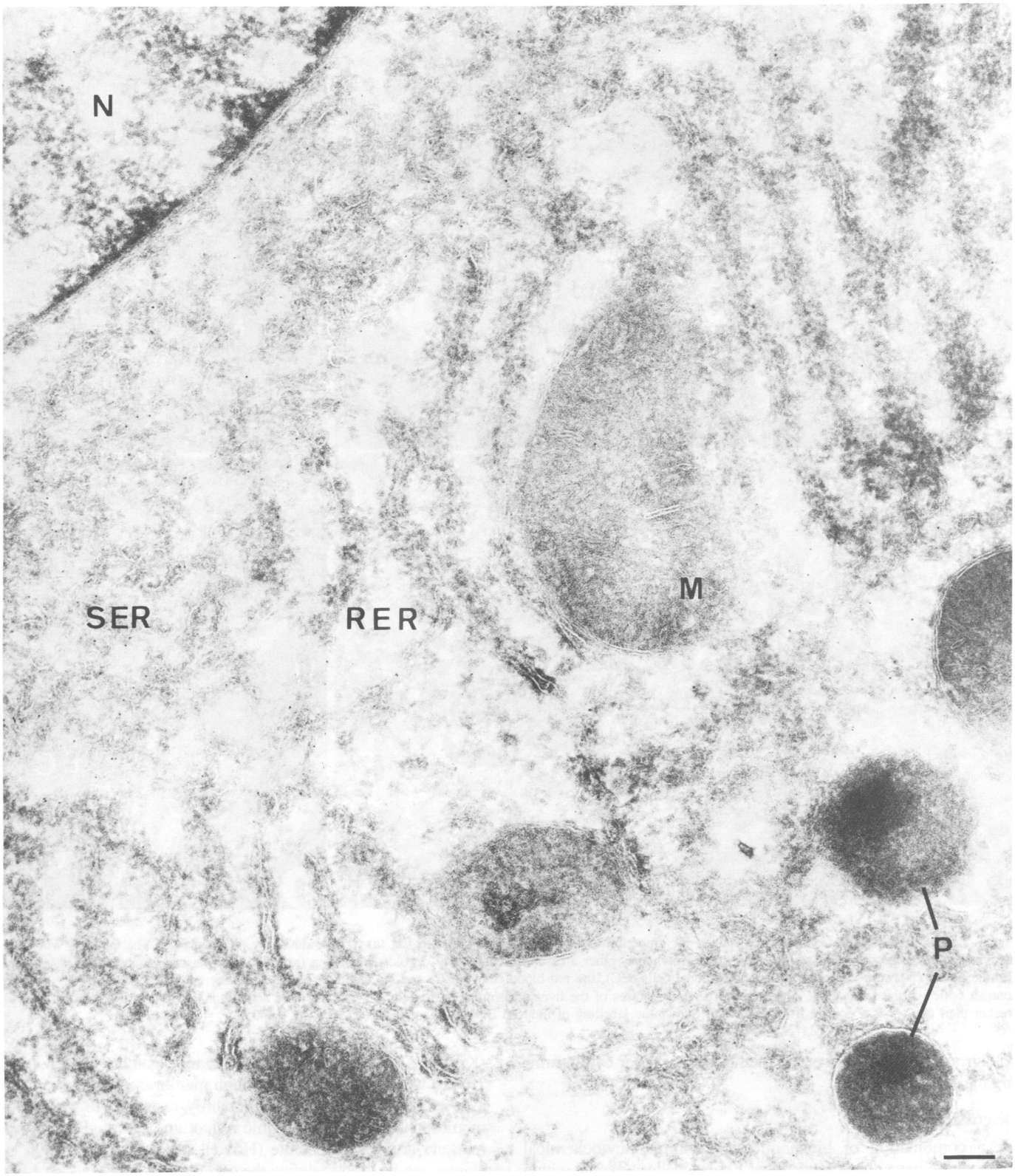

Fig. 4. Electron micrograph of a frozen thin section from control rat liver stained with antibodies to EH and protein A-gold (12 nm). The labelling was concentrated over the membrane of the SER. The RER, the mitochondria (M), the peroxisomes (P) and the nucleus (N) were not labelled. × 46 500; bar = 0.2 μm.

with the subcellular fractions highly enriched in SER membranes. A low EH activity was present, however, in the high density gradient fractions, rich in RNA containing mainly RER vesicles. 10–15% of the EH activity was associated with the fractions denser than 1.17. These values are an upper limit of the actual amount of the newly synthesized enzyme present within the RER since the fractions are clearly contaminated by some SER vesicles. Our immunolabelling data indicate that this actual amount is much

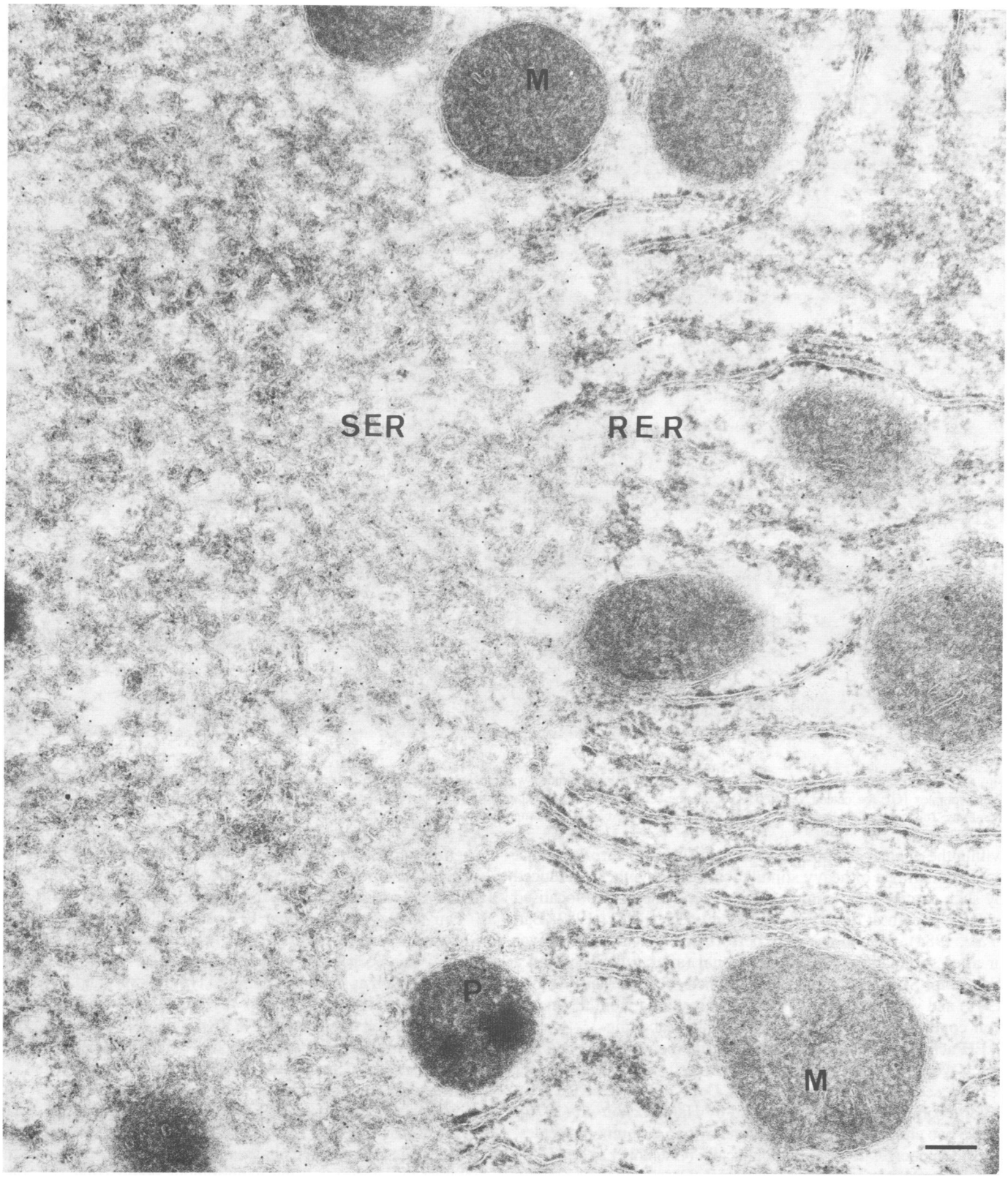

Fig. 5. Electron micrographs of a frozen thin section from rat liver induced with phenobarbital, stained with antibodies to EH and protein A-gold (12 nm). The labelling was restricted to the membranes of the SER accumulated in specific areas of the cytoplasm. The RER, the mitochondria (M) and the peroxisomes (P) were not labelled. × 46 500; bar = 0.2 μm.

lower and therefore that EH is a good marker for SER membranes. The situation could be different during phases of rapid synthesis of the enzyme, for example during the first 2 days of the phenobarbital induction. In fact EH appears to be localised both in the smooth and the rough ER in rat liver induced by 2-acetylaminofluorene (Novikoff *et al.*, 1979; Waechter *et al.*, 1982). EH activity was also found in rat liver nuclei after 3-methylcholanthrene treatment (Bornstein *et al.*, 1979) (probably

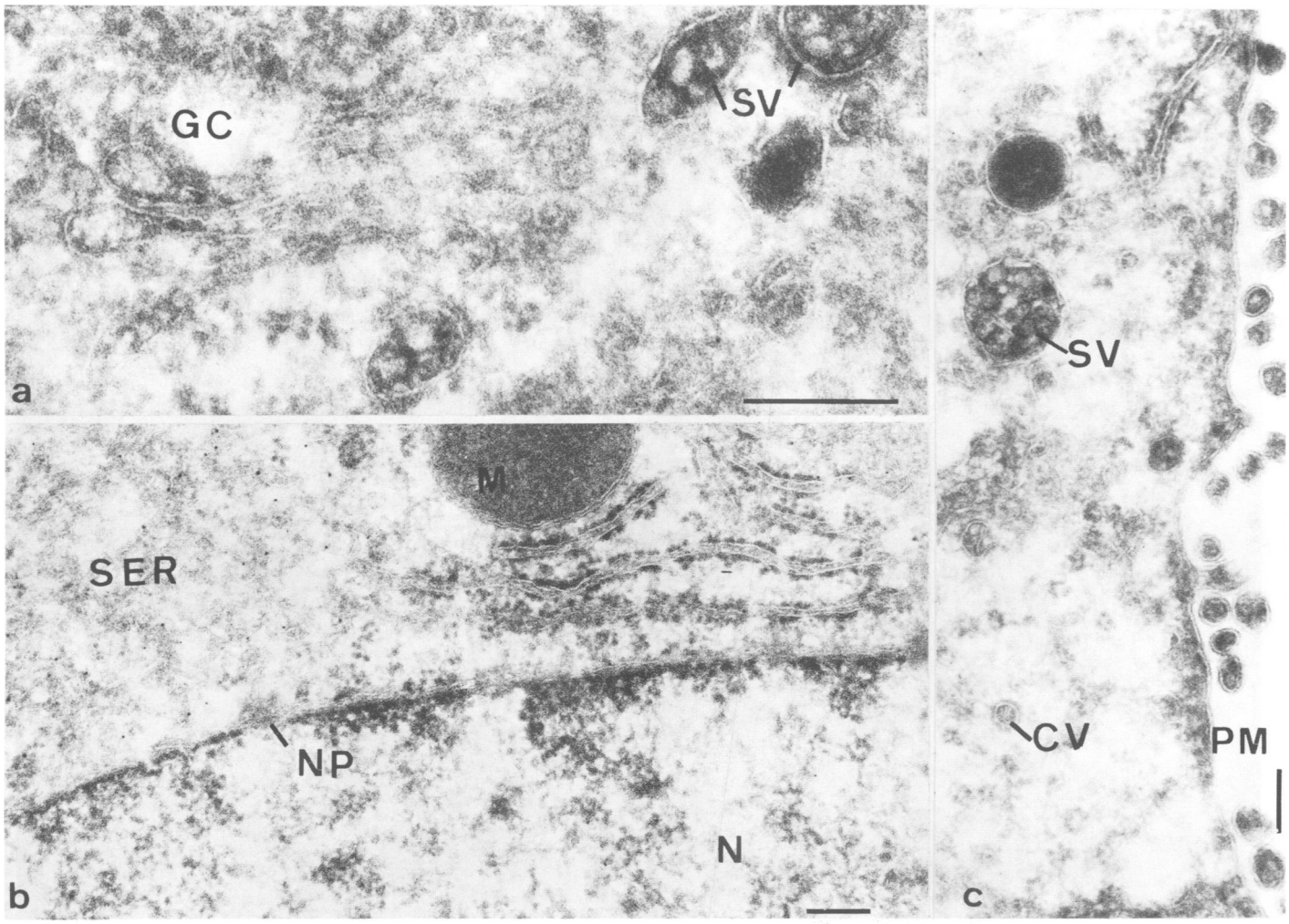

Fig. 6. Electron micrographs of frozen thin section from rat liver induced with phenobarbital, stained with antibodies to EH and protein A-gold (12 nm). The Golgi complex (G), the coated vesicles (CV) and the secretory vesicles containing VLDL **(a)** the plasma membranes (PM) **(b)** and the nucleus including its membrane **(c)** were unlabelled. **a** × 112 000; **b,c** × 46 500; bar = 0.2 μm.

within the nuclear membrane cisternae). It is not surprising that under conditions of heavy stimulation of EH synthesis, induced by such carcinogenic compounds, this enzyme is also localised in RER (Levin *et al.*, 1978; Kamdem *et al.*, 1981, 1982; Batt *et al.*, 1984). Our studies were performed either in normal liver or after stimulation with phenobarbital (after 5 days), when a plateau for SER development was reached (Bolender and Weibel, 1979) and when the only modification resulting from the induction conditions was a net increase of the labelling.

EH activity was first reported as being membrane-bound and much work has subsequently been devoted to this enzyme (for review, see Oesch, 1973; Jerina and Daly, 1974; Oesch, 1979). Later a soluble molecule was found by Hammock *et al.* (1976). These two enzyme activities are borne by two different molecules. They do not cross-react immunologically (Guenthner *et al.*, 1981) and their substrate specificity is different (Oesch and Golan, 1980; Wang *et al.*, 1982b). Moreover EH-specific activities and the levels of their induction by phenobarbital differ considerably depending on the substrate used to measure them (Bornstein *et al.*, 1979; Gonzalez and Kasper, 1982).

A soluble-like EH activity was also characterised in purified fractions of mouse liver peroxisomes (Waechter *et al.*, 1983) or mitochondria (Gill and Hammock, 1981). Our immunological analysis of soluble and membrane fractions and our immunochemistry data indicate clearly that we are dealing exclusively with the membrane-bound molecule. No significant labelling was found over the cytosol or any organelle other than SER.

Our studies showing that EH could be used as a marker of SER membrane in rat liver are of widespread interest since EH activity has been detected in every tissue so far tested (Oesch, 1979), as well as in human cultured lymphocytes and fibroblasts (Glatt *et al.*, 1980). Moreover preliminary results indicate that in different cell lines such as NRK (normal rat kidney) and BHK (baby hamster kidney) our antibodies to EH label a well-developed reticulated network (unpublished results). This network was clearly different from that stained with antibodies to the RER membranes (Louvard *et al.*, 1982), in particular the nuclear membrane is not labelled. Experiments are underway to determine if the tissue cultured cells possess EH activity. It would be great interest to determine if the membranous compartments defined by this antigen are implicated in the transport of secretory and/or membrane proteins.

Materials and methods

Animals

Male Sprague Dawley rats weighing 200–220 g were used. Sodium phenobarbital (75 mg/kg/day in 0.9% NaCl) was injected i.p. This treatment was applied

during five consecutive days and the rats were killed by decapitation on the 6th day. Control rats received 0.9% NaCl in the same conditions.

Epoxide hydrolase purification

EH from phenobarbital-treated rat liver was purified according to Knowles and Burchell (1977) with the following modifications. After chromatographic separation on DEAE and CM cellulose, the fractions containing EH were pooled and applied on phenyl-Sepharose and then on DEAE-Sephadex. Excess detergent was removed by Biobeads (Biorad, France) chromatography.

Antibodies

0.5 mg of purified protein was injected s.c. into the neck area of rabbits (Fauve de Bourgogne, local supplier). Booster injections were made the same way after 2 weeks, i.m. the 4th week and i.v. (0.1 mg) the 11th week. The animals were bled after 12 weeks.

The serum was affinity purified on the purified enzyme, immobilized on Sepharose Ultrogel (IBF, France) according to Ternynck and Avrameas (1976). The specificity of the purified antibodies was tested on different fractions after electrophoresis on polyacrylamide gel in the presence of SDS (Laemmli, 1970) and immunoelectrotransfer on nitrocellulose (Burnette, 1981). The antibodies were also affinity purified on nitrocellulose strips after electrotransfer (Olmsted, 1981).

Subcellular fractionation

Rats were fasted for 12 h and killed by decapitation. The livers were rapidly perfused with 0.9% NaCl and excised. The homogenate and its first subsequent differential centrifugation were done according to the procedure of Morré (1972). The supernatant obtained, mainly devoid of Golgi, nuclear and plasma membranes, was then separated from mitochondria by a 11 000 *g* centrifugation for 10 min. It was then subfractionated on a discontinuous sucrose gradient for 3 h at 100 000 *g* in a SW 28 rotor. We used a series of five density layers for collecting four fractions, respectively, at borderlines of 0.25/0.81 M, 0.81/1.02 M, 1.02/1.21 M, 1.21/1.30 M sucrose and the pellet. The corresponding densities were 1.03/1.10, 1.10/1.13, 1.13/1.15, 1.15/1.17 and > 1.17. Each fraction was centrifuged at 100 000 *g* for 30 min in order to pellet the membranes which were resuspended in buffer (sucrose 0.5 M, Tris 0.05 M, $MgCl_2$ 0.005 M).

Assays

EH activity was measured by the technique of Dansette *et al.* (1979) using benzo-[a]pyrene-4,5-oxide as substrate. This enzymatic activity is defined as mEH_b according to Batt *et al.* (1984). UDPGT activity was determined according to Mulder and Van Doorn (1975) adapted to the centrifugal analyser (Colin-Neiger *et al.*, 1984), using 4-methylumbelliferone as substrate. RNA was measured by the method of Fleck and Begg (1965) and proteins according to Lowry *et al.* (1951).

Immunocytochemistry

The livers were fixed with 2% formaldehyde in 0.1 M phosphate buffer pH 7.4 for light microscopy studies and 2% formaldehyde, 0.5% glutaraldehyde for electron microscopy. Frozen sections were prepared according to Tokuyasu (1973) and stained with the positive negative staining described by Griffith *et al.* (1983). Probes were sheep anti-rabbit IgG complexed with rhodamin (Brandtzaeg, 1976) or protein A-gold (12 nm).

Tissue-cultured cells were fixed with 3% formaldehyde in PBS containing 10 μM Ca^{2+} and Mg^{2+} and stained with antibodies to EH according to Wang *et al.* (1982a).

Reagents

Glutaraldehyde was obtained from Ladd Research Industries, Burlington, USA; diaminobenzidine hydrochloride from Sigma, sheep anti-rabbit IgG conjugated to HRP from Institut Pasteur, France.

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