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Immuno-electron-microscopic studies on the subcellular distribution of rat liver epoxide hydrolase and the effect of phenobarbitone and 2-acetamidofluorene treatment

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The distribution of rat liver epoxide hydrolase in various subcellular fractions was investigated by immuno-electron-microscopy. Ferritin-linked monospecific anti-(epoxide hydrolase) immunoglobulins bound specifically to the cytoplasmic surfaces of total microsomal preparations and smooth and rough microsomal fractions as well as the nuclear envelope. Specific binding was not observed when the ferritin conjugates were incubated with peroxisomes, lysosomes and mitochondria. The average specific ferritin load of the individual subcellular fractions correlated well with the measured epoxide hydrolase activities. This correlation was observed with fractions prepared from control, phenobarbitone-treated and 2-acetamidofluorene-treated rats.

Epoxide hydrolase (EC 3.3.2.3) catalyses the conversion of epoxides and arene oxides into the corresponding *trans*-dihydrodiols. Epoxide hydrolase activity is found in both membrane and cytoplasmic subcellular fractions; however, the observed distribution depends on the substrate used (Ota & Hammock, 1980; Oesch & Golan, 1980). The microsomal enzyme has been extensively studied (Oesch, 1973; Lu & Miwa, 1980); it is known to catalyse the hydrolysis of a wide variety of arene oxides and alkene oxides, and is also involved in the metabolic activation of some polycyclic aromatic hydrocarbons (Wood *et al.*, 1976; Bentley *et al.*, 1977). Epoxide hydrolase activity has been observed in smooth and rough microsomal fractions, nuclear membrane, plasma membrane and Golgi apparatus (Jernström *et al.*, 1976; Fahl *et al.*, 1978; Mukhtar *et al.*, 1978; Thomas *et al.*, 1979; Stasiecki *et al.*, 1980). An enzyme with properties similar to that in cytoplasmic fractions has been reported in mitochondria (Gill & Hammock, 1981), but the enzymes in other membranous subcellular fractions are similar to the microsomal enzyme. In fact, the enzymes in nuclear and microsomal fractions have very similar immunological properties (Thomas *et al.*, 1979).

These studies of epoxide hydrolase distribution have all involved measurement of enzyme activity in isolated subcellular fractions that are usually contaminated by microsomal membranes. Thus it is often not possible to disprove that the low specific activities measured resulted from this contamination, since the properties of epoxide hydrolase in microsomal and non-microsomal membrane fractions appear to be similar. The association of this enzyme with specific cellular structures can be demonstrated by ferritin-linked anti-(epoxide hydrolase) antibodies, which, when observed in the electron microscope, allow simultaneous identification of the structures and quantitative assessment of their enzyme content. Ferritin immuno-electron-microscopy also has the advantage that the inhomogeneous distribution of an enzyme within a subcellular fraction may be observed. This is of interest, since experiments with deoxycholate-treated microsomal fractions indicated that epoxide hydrolase activity was not uniformly distributed among the microsomal vesicles (Stasiecki *et al.*, 1979).

Qualitative and quantitative aspects of the subcellular distribution of NADPH–cytochrome *c* reductase (Morimoto *et al.*, 1976; Aoi *et al.*, 1981), cytochrome b_5 (Fowler *et al.*, 1976; Remacle *et al.*, 1976), cytochrome *P*-450 (Matsuura *et al.*, 1978, 1979) and NADH–cytochrome b_5 reductase (Schulze *et al.*, 1978) have been studied by immuno-electron-microscopy. In the present study we have investigated the distribution of epoxide hydrolase in various rat liver subcellular fractions with ferritin conjugates of antibodies raised against purified microsomal epoxide hydrolase.

Abbreviation used: IgG, immunoglobulin G.

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Our aim was to study the distribution of epoxide hydrolase within the endoplasmic reticulum and to demonstrate that the nuclear enzyme activity was really associated with the nuclear membrane. Finally, the association of epoxide hydrolase with lysosomes and peroxisomes, which may be easily obtained in a crude fraction but are difficult to prepare free of contamination by other subcellular fractions, was investigated.

Experimental

Chemicals

[^{14}C]Styrene oxide (1.23 μCi/mg) was synthesized as described by Oesch *et al.* (1971). Horse spleen ferritin (6 times crystallized; cadmium-free) was obtained from Miles Laboratories, Elkhart, IN, U.S.A. All other chemicals were of analytical or reagent grade.

Animals

Male albino Sprague–Dawley rats (180–250g body wt.) were used. When required, enzyme activity was induced by oral administration of phenobarbitone (three daily doses of 80mg/kg), or by addition of 2-acetamidofluorene (500p.p.m.) to the basal diet, which was given for 7 days. In all experiments food was withdrawn 24h before animals were killed by exsanguination. The livers were removed and chilled on ice. Homogenates of two or three livers were then combined for preparation of the various liver subcellular fractions.

Preparation of a ribosome-free microsomal fraction

A washed microsomal fraction was prepared as previously described (Schmid *et al.*, 1980). The ribosomes were removed by treatment of the microsomal fraction with pyrophosphate, followed by chromatography on Bio-Gel A-150m (50–100 mesh; Bio-Rad Laboratories, Richmond, CA, U.S.A.) (Remacle *et al.*, 1976). This procedure decreased the RNA content to about 20%, as determined biochemically (Schneider, 1961). Electron micrographs revealed that these fractions were essentially composed of smooth-surfaced vesicles.

Preparation of smooth and rough microsomal fractions

Smooth and rough microsomal fractions were isolated by the method of Boulan *et al.* (1978). For the experiments reported in the present paper, the RM_2 layer was used as the source of rough microsomal membranes. The RNA contents were 223 and 38 μg/mg of protein ($\pm$10%) for the rough and smooth microsomal fractions respectively. The fractions were then treated with pyrophosphate as described above (Remacle *et al.*, 1976).

Preparation of ribosome-free nuclei

Liver cell nuclei were prepared as described by Franke *et al.* (1976). The nuclear pellet was resuspended in 25mM-sodium pyrophosphate buffer, pH7.4, containing 0.25M-sucrose and 0.2g of NaN_3/l, and left for 30min at 4°C. The nuclei were re-collected by centrifugation at 2000rev./min for 5min at 4°C (Sorvall RC-2 centrifuge, SS-34 rotor).

Preparation of a mitochondrial, lysosomal and peroxisomal fraction (MLP fraction)

Fractions containing mitochondria, lysosomes and peroxisomes were isolated as outlined by Smith *et al.* (1966). The purified fractions were treated with pyrophosphate (as described above), to enable comparison with the other subcellular fractions. After this treatment the organelles were pelleted at 8500rev./min for 10min at 4°C (Sorvall RC-2 centrifuge, SS-34 rotor).

Purification of rat liver microsomal epoxide hydrolase

Epoxide hydrolase was isolated as described previously (Bentley & Oesch, 1975). Enzyme activity was determined with styrene oxide as substrate (Oesch *et al.*, 1971). Protein was determined by the method of Lowry *et al.* (1951), with serum albumin as the standard.

Preparation of monospecific antibodies

Antibodies were raised in a goat against purified rat liver microsomal epoxide hydrolase and processed as described by Bentley *et al.* (1979). The specificities of antisera, IgG fractions and monospecific antibodies were tested by double-diffusion immunoprecipitation. Control experiments were performed with IgG isolated from pre-immune goat serum by DEAE-cellulose chromatography (Fahey, 1967).

Preparation of IgG–ferritin conjugates

Pre-immune goat IgG and monospecific goat anti-(rat epoxide hydrolase) IgG were covalently bound to ferritin, with glutaraldehyde as the coupling reagent (Kishida *et al.*, 1975). Before its use, ferritin was chromatographed on Bio-Gel A-5m (200–400 mesh; Bio-Rad Laboratories). The IgG–ferritin conjugates were separated from cross-linked ferritin polymers by filtration through Bio-Gel A-5m; the elution was monitored by absorption measurements at 280 and 360nm. The fractions containing the IgG–ferritin conjugates (and possibly some free ferritin) were collected, concentrated by ultrafiltration (Amicon PM 10 membrane) and tested for specificity by immunodiffusion.

Incubations of membrane fractions with IgG–ferritin conjugates

The various cell fractions were incubated with the goat anti-(rat epoxide hydrolase) IgG–ferritin conjugate (specific conjugate) for 90min at 4°C. For control experiments a pre-immune goat IgG–ferritin conjugate (non-specific conjugate) was used. The incubation mixture (2–3ml) contained 1% (w/v) Triton WR-1339, 0.3M-NaCl, 0.25M-sucrose, 20mM-Tris/HCl buffer, pH7.4, and 0.2g of NaN_3/l (Remacle *et al.*, 1976). Triton WR-1339 was omitted when nuclear or MLP fractions were incubated. The protein concentrations in the incubation medium were 0.1–0.3mg/ml for subcellular fractions and 0.2–0.6mg/ml for the ferritin–IgG conjugates. The amount of conjugate used was always sufficient to give a more than 5-fold molar excess over epoxide hydrolase. [The molar content of epoxide hydrolase may be estimated from the specific activity of the enzyme in the membrane fraction and that of the purified enzyme, by assuming a molecular weight of 49000 (Bentley & Oesch (1975)].

Separation of IgG–ferritin conjugates that had not reacted

IgG–ferritin conjugates that had not reacted were separated from microsomal vesicles by chromatography on Bio-Gel A-150m (50–100 mesh) as outlined by Remacle *et al.* (1976). Nuclei that had reacted were separated from unbound conjugate by centrifugation through a linear sucrose gradient (0.45–1.25M, overlaying a cushion of 1.65M-sucrose) at 60000*g* (Beckman SW 41 rotor) for 1h (Matsuura *et al.*, 1978). The labelled membrane fractions containing mitochondria, lysosomes and peroxisomes were centrifuged through a discontinuous sucrose gradient (0.45M, 0.6M and 0.88M) at 25000*g* for 1h (Beckman SW 41 rotor).

Electron microscopy

Fractions containing the immuno-labelled microsomal membranes (2ml, containing approx. 150μg of protein) were fixed in 2% (v/v) glutaraldehyde in 50mM-phosphate buffer, pH7.4 (5ml), for 30min at 4°C. The microsomal vesicles were then collected on Millipore VSWP filters (pore size 0.025μm) by a modification (Stäubli *et al.*, 1977) of the procedure of Baudhuin *et al.* (1967). After two washes with 50mM-phosphate buffer, pH7.4, the Millipore filters with the adhering pellicles were removed from the filter unit and post-fixed for 30min at 4°C with 1% (w/v) OsO_4 in phosphate buffer (Millonig, 1961). After a short wash in Millonig's buffer, the preparations were dehydrated in graded concentrations of ethanol and the Millipore filters were dissolved in propylene oxide. Samples were embedded in Spurr's low-viscosity embedding medium (Spurr, 1969), in flat moulds. To minimize the influence of section thickness on the quantitative measurements (see below), each pair of pellicles obtained from specifically and non-specifically labelled microsomal preparations was embedded in the same mould. Thin sections (approx. 80nm thick) were made, collected on slotted grids and double-stained with uranyl acetate and lead citrate (Reynolds, 1963). The pellets containing nuclear and MLP fractions that had reacted (see above) were fixed, embedded and sectioned as outlined for the microsomal pellicles. Photomicrographs of cross-sectioned microsomal pellicles and the other fractions were taken at a magnification of 20000 in a Philips EM 300 electron microscope; the accuracy of the magnification was checked by means of a carbon grating replica (2160 lines/mm; Balzers Union, Balzers, Lichtenstein). The negatives were enlarged to a final magnification of 100000 on Kodagraph Transtar TP5 paper (32cm × 44cm).

Quantitative analysis of microsomal fractions

For the quantitative assessment of the binding of anti-(epoxide hydrolase) IgG–ferritin conjugates, only perfect cross-sections of microsomal vesicles were selected, i.e. the boundary of the microsomal profile had to be well defined over the whole perimeter. Each of the selected profiles was numbered and the length of its perimeter was determined by means of a semi-automatic image analyser (MOP/AM 02; Kontron, Zürich, Switzerland). The ferritin images associated with the surface of the individual profiles were counted, whereby a ferritin particle was judged to be attached to a particular profile if it fulfilled the condition of closest contact (Remacle *et al.*, 1976). For quantitative analysis of total microsomal fractions, 11820 perfectly cross-sectioned vesicles were used, and 8618 vesicles for analysis of smooth and rough microsomal fractions. Vesicles were assigned to one of six perimeter classes according to their perimeter length.

To compare the morphological data with the measured epoxide hydrolase activity, an average ferritin load per μm perimeter length was calculated for each experimental group by using eqn. (1):

$$\text{Average ferritin load} = \frac{\sum_{i=1}^{n} m_i}{\sum_{i=1}^{n} l_i} \qquad (1)$$

where m_i = the number of ferritin particles on the ith profile, l_i = the perimeter length (μm) of the ith profile, and n = the number of profiles investigated in an experimental group.

The specific ferritin load was then calculated by subtracting the average ferritin load of the immuno-

logical control incubations from that of the incubations with anti-(epoxide hydrolase) conjugates. This calculation assumes that ferritin labelling by the specific conjugates is a composite of specific and non-specific binding, whereby these two processes are considered to be independent of each other.

Quantitative analysis of nuclear fractions

Ferritin images were counted only when they were associated with morphologically well-defined nuclear envelope (total length: 629 μm). The results were expressed as the average number of ferritin particles per μm membrane length.

Results

Subcellular distribution of epoxide hydrolase activity

The specific epoxide hydrolase activities in the isolated subcellular fractions that were used for immuno-electron-microscopic analysis are shown in Table 1. The activity in microsomal fractions was increased by pretreatment both with phenobarbitone and with 2-acetamidofluorene. Nuclear epoxide hydrolase activity was also higher after 2-acetamidofluorene pretreatment. Rough and smooth microsomal fractions isolated from untreated rats had similar specific epoxide hydrolase activities, which were increased to a similar extent by acetamidofluorene treatment.

Specificity of antibodies

As shown in Fig. 1(*a*), a single precipitation line was observed when the IgG fraction was tested by immunodiffusion against purified rat liver epoxide hydrolase. The specific IgG–ferritin complexes also yielded a single precipitation line when tested similarly (Fig. 1*b*).

Immuno-electron microscopy

Qualitative observations. When ribosome-free microsomal fractions were incubated with specific ferritin conjugates, the outer surface of most microsomal profiles was labelled with one or more ferritin particles, as shown in Plate 1(*a*), for microsomal fractions from an untreated rat. In contrast, control incubations with unspecific conjugates resulted in much less ferritin labelling (Plate 1*b*). The binding of ferritin particles to the luminal surface of microsomal vesicles was not observed. The ferritin load of microsomal fractions from rats pretreated with 2-acetamidofluorene was visibly increased when microsomal fractions were incubated with specific conjugates (Plate 1*d*), but not when microsomal fractions from phenobarbitone-induced animals were used (Plate 1*c*). In this latter case the enzyme induction was apparent only from the quantitative analysis (see below). Unspecific ferritin binding was not affected by the enzyme inducers.

Qualitatively, the results obtained with ribosome-free rough and smooth microsomal fractions were similar to those obtained with unfractionated microsomal preparations (results not shown). Both rough and smooth microsomal vesicles were labelled exclusively on the outer surfaces when incubated with specific ferritin conjugates; the extent of labelling of both fractions appeared similar on visual inspection. Fractions incubated with unspecific conjugates were less extensively labelled. The extent of binding of specific ferritin conjugates to both rough and smooth fractions was markedly increased by pretreatment of rats with 2-acetamidofluorene.

Electron micrographs of ribosome-free nuclei prepared from either control or 2-acetamidofluorene-treated animals and incubated with specific conjugates are shown in Plates 1(*e*) and 1(*f*) respectively. The cytoplasmic face of the nuclear envelopes was studded with ferritin molecules. The degree of labelling was clearly higher with nuclei from 2-acetamidofluorene-treated rats. The unspecific ferritin conjugates bound less extensively to the nuclear envelope (results not shown). The nuclear inner membrane and the nuclear matrix were both almost free of ferritin particles, whether incubations were performed with specific or non-specific conjugates.

Electron micrographs of the MLP fraction revealed that very few ferritin particles were bound

EXPLANATION OF PLATE 1

Electron micrographs showing ferritin particles associated with either microsomal vesicles or parts of the nuclear membrane

(*a*) Microsomal fraction isolated from control rats and incubated with specific ferritin conjugates. (*b*) Microsomal fraction isolated from control rats and incubated with unspecific ferritin conjugates (immunological control). (*c*) Microsomal fraction isolated from phenobarbitone-treated rats and incubated with specific ferritin conjugates. (*d*) Microsomal fraction isolated from acetamidofluorene-treated rats and incubated with specific ferritin conjugates. (*e*) Part of a nucleus isolated from an untreated rat and incubated with specific ferritin conjugate. (*f*) Part of a nucleus isolated from an acetamidofluorene-treated rat and incubated with specific ferritin conjugates. Note that ferritin images are associated only with the outer surface of the microsomal profiles and with the outer leaflet of the nuclear envelope. The number of ferritin particles associated with the membrane was greater when specific ferritin conjugates were used than when unspecific ferritin conjugates (immunological control) were used (compare *a* and *b*). Magnification $\times 100\,000$.

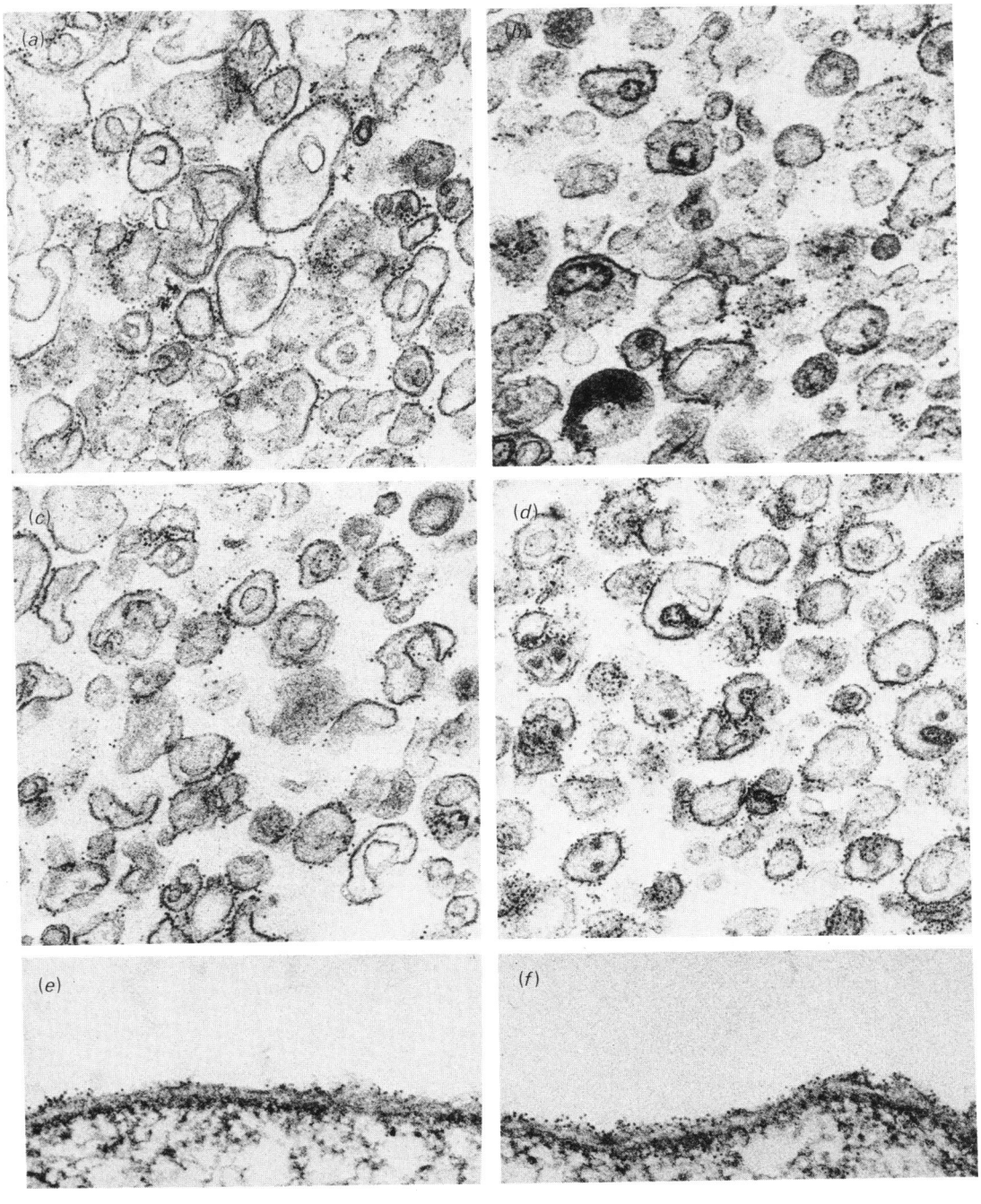
(a)
(b)
(c)
(d)
(e)
(f)

Table 1. *Subcellular distribution of epoxide hydrolase*

Enzyme activity was measured with styrene oxide as substrate. Results are expressed as nmol of styrene glycol formed/min per mg of protein. For induction experiments, animals were pretreated as described in the Experimental section. N.D., Not detectable.

Subcellular fraction	Specific epoxide hydrolase activity		
	Control	Phenobarbitone-treated	2-Acetamidofluorene-treated
Unfractionated microsomal preparation	16.6	26.6	54.6
Smooth microsomal fraction	13.8	—	46.0
Rough microsomal fraction	13.7	—	55.1
Nuclear fraction	0.88	—	1.25
MLP fraction	N.D.	0.51	—

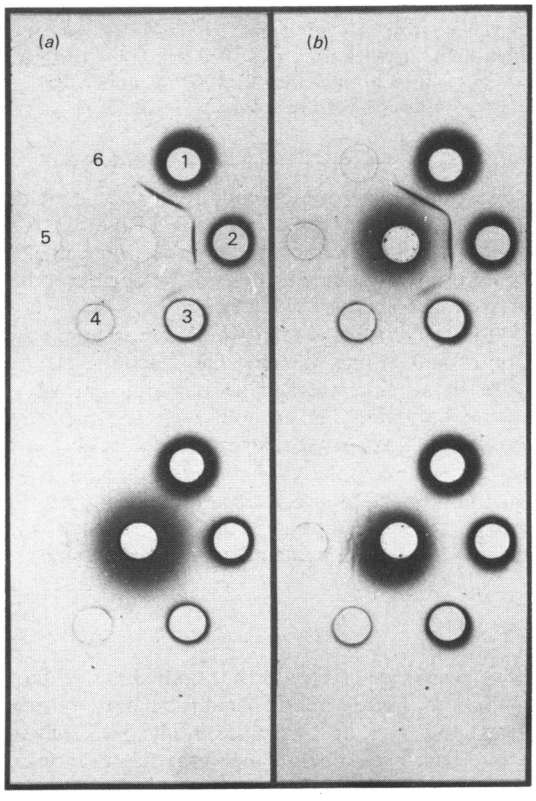

Fig. 1. *Ouchterlony double-diffusion patterns*

Wells 1, 2, 3, 4, 5 and 6 contained 5 μl of purified rat epoxide hydrolase (2.4, 1.2, 0.6, 0.3, 0.15 and 0.075 mg of protein/ml). The centre wells contained 5 μl of monospecific goat anti-(rat epoxide hydrolase) IgG [2.4 mg/ml, (*a*) top], pre-immune goat IgG [5.5 mg/ml, (*a*) bottom], goat anti-(rat epoxide hydrolase) IgG–ferritin conjugate [12 mg/ml, (*b*) top] and pre-immune goat IgG–ferritin conjugate [15 mg/ml, (*b*) bottom].

to mitochondria and lysosomes, whether they were incubated with specific or non-specific conjugates (results not shown). The peroxisomes were more heavily labelled, but no difference in the degree of binding was observed between incubations with specific conjugates and the immunological controls.

Quantitative analysis. The distribution of microsomal vesicles in the six perimeter classes was similar in all experimental groups (unfractionated microsomal preparations; rough and smooth microsomal fractions from control and induced animals), as shown in Fig. 2 for unfractionated microsomal preparations. In each experimental group the average ferritin load increased with increasing perimeter length of the vesicles from unfractionated microsomal preparations (Fig. 3) and rough microsomal fractions (Fig. 4). This increase was less apparent in the two smallest-perimeter classes, but was approximately proportional to vesicle size in classes 3–6 for unfrationated microsomal preparations and rough microsomal fractions. Smooth microsomal fractions showed less binding than expected to vesicles of the largest-perimeter classes (Fig. 4*a*). In all except the smallest vesicles, the average ferritin load was markedly increased when microsomal preparations from induced animals were incubated with specific conjugates (Figs. 3 and 4*b*). However, non-specific binding was not affected by enzyme induction (Figs. 3 and 4).

The specific average ferritin loads of the individual subcellular fractions are summarized in Table 2. The values were calculated from all the available data. However, for unfractionated microsomal preparations and smooth and rough microsomal fractions similar results were obtained when data from perimeter classes 1 and 2 or classes 1, 2 and 6 were omitted from the calculations. The specific average ferritin loads of unfractionated microsomal preparations and smooth and rough microsomal fractions from control rats were all very similar, which is

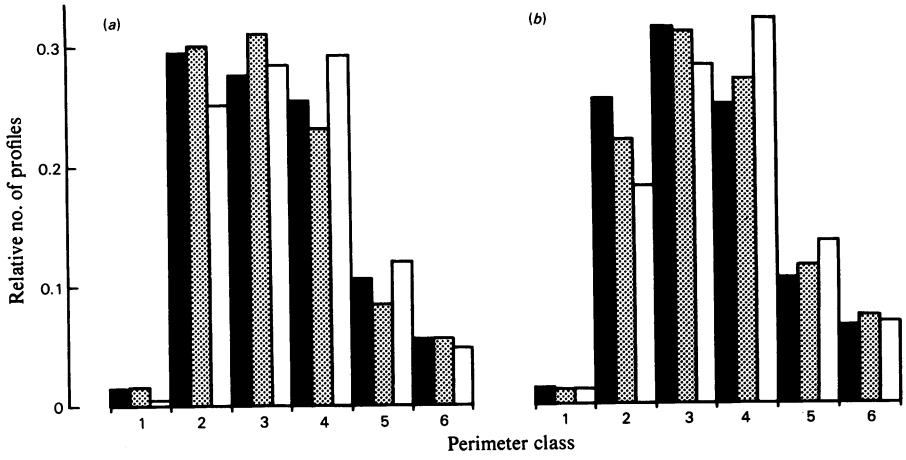

Fig. 2. *Perimeter-length distribution of microsomal profiles*

The relative number of profiles is defined as $\Delta N/N$, where N is the total number of profiles per experimental group subjected to quantitative analysis. Total microsomal fractions were investigated from untreated (■), phenobarbitone-treated (▩) and 2-acetamidofluorene-treated (□) animals. The profiles were classified according to their perimeter length into six perimeter classes: class 1, perimeter length 0–120nm; class 2, 120–240nm; class 3, 240–360nm; class 4, 360–480nm; class 5, 480–600nm; class 6, >600nm. Ferritin–antibody incubations were performed with (*a*) specific or (*b*) unspecific conjugate.

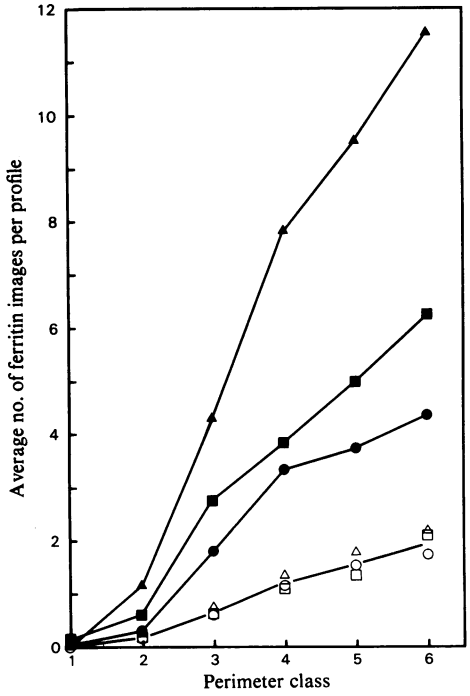

Fig. 3. *Relationship between the average number of ferritin particles bound per microsomal profile and the perimeter length of microsomal fractions*

The perimeter classes are defined in Fig. 2. Total microsomal fractions from untreated (● and ○), phenobarbitone-treated (■ and □) and 2-acetamidofluorene-treated animals (▲ and △) were incubated with specific (●, ■ and ▲) or unspecific (○, □ and △) conjugate.

in agreement with the epoxide hydrolase activities measured in these three fractions (Table 1). The specific average ferritin load of the outer nuclear membrane was similar to that of the corresponding microsomal fractions (Table 2).

With unfractionated microsomal preparations and smooth and rough microsomal fractions the increase in specific average ferritin load caused by enzyme induction agreed well with the measured increase in epoxide hydrolase activity, as shown in Fig. 5. However, the epoxide hydrolase induction measured by immuno-electron-microscopy in the nuclear outer membrane was higher than that expected from the enzyme-activity measurement.

Discussion

The results demonstrate that conjugates of ferritin and antibodies raised against highly purified microsomal epoxide hydrolase bind specifically to the outer surfaces of some membranous fractions of the cell and that the extent of the binding (ferritin images/μm) was proportional to the epoxide hydrolase activity of the corresponding subcellular fractions. This observation indicates that some antigenic determinants of the epoxide hydrolase molecule are accessible from the cytoplasmic surface of the membranes and that the enzyme is not totally embedded within the phospholipid bilayer, as was suggested for microsomal vesicles by Seidegård *et al.* (1978). The specific average ferritin load of unfractionated microsomal preparation was 3.7 images/μm perimeter length (Table 2); thus, taking

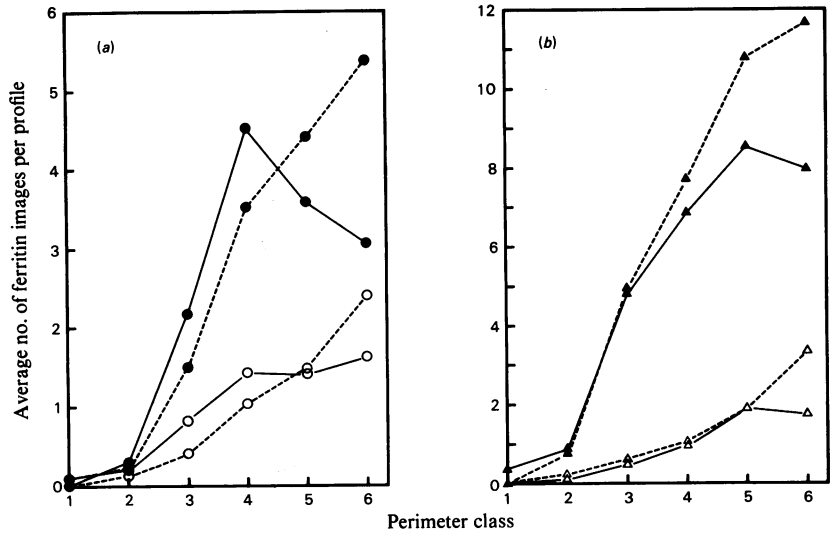

Fig. 4. *Relationship between the average number of ferritin particles bound per microsomal profile and the perimeter length of smooth and rough microsomal fractions*

A definition of the perimeter classes is given in Fig. 2. Smooth (——) and rough (– – – –) microsomal subfractions were obtained from (*a*) untreated or (*b*) 2-acetamidofluorene-treated animals and incubated with specific (● and ▲) or unspecific (○ and △) conjugate.

Table 2. *Average ferritin loads of the different experimental groups*

Average ferritin loads were calculated as described in the Experimental section and are expressed as ferritin images/μm membrane length. The values in parentheses show the average extent of non-specific binding in the individual groups, which has been subtracted from the specific binding values shown in the Table.

Treatment	Group ...	Average specific ferritin load/nm membrane length: Unfractionated microsomal preparation	Smooth microsomal fraction	Rough microsomal fraction	Nuclear fraction
Control		3.7 (2.3)	4.5 (2.8)	4.1 (2.4)	5.5 (2.4)
Phenobarbitone-treated		5.8 (2.3)	—	—	—
2-Acetamidofluorene-treated		12.6 (2.9)	12.3 (2.3)	14.0 (2.7)	16.3 (1.1)

into account a section thickness of about 0.08 μm, an average ferritin load of 46.2 images/μm² may be calculated. The specific epoxide hydrolase activity of the microsomal fraction used for these incubations was 16.6 nmol/min per mg of protein. The specific activity of apparently pure rat hepatic microsomal epoxide hydrolase has been reported to be approx. 700 nmol/min per mg of protein (Bentley & Oesch, 1975; Du Bois *et al.*, 1978). Thus the enzyme should account for approx. 2% of the microsomal protein. The number of epoxide hydrolase molecules/μm² microsomal area may be calculated on the basis of a molecular weight of 49000 (Bentley & Oesch, 1975) and various estimations of the protein content per unit microsomal area (Weibel *et al.*, 1969; Schulze & Staudinger, 1971; Ito, 1974; Morimoto *et al.*, 1976). The value obtained ranges from 382 to 1520 molecules/μm² membrane area, which indicates that only 3–12.5% of the enzyme molecules are accessible for the ferritin–antibody conjugates. A similar extent of labelling has been observed for cytochrome b_5 (Remacle *et al.*, 1976).

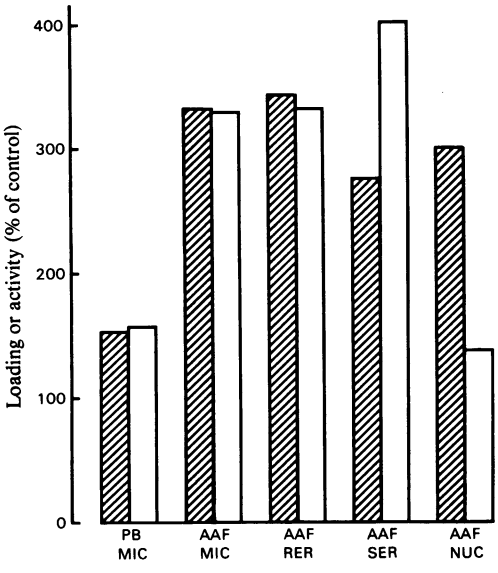

Fig. 5. *Perimeter-length-specific conjugate binding* (▨) *and styrene oxide hydrolase activity* (□) *of different subcellular fractions obtained from rats pretreated with phenobarbitone (PB) or 2-acetamidofluorene (AAF)*
Results are given as percentages of the corresponding control values. The conjugate-binding data are corrected for non-specific adsorption. Abbreviations: MIC, total microsomal preparation; RER, rough microsomal fraction; SER, smooth microsomal fraction; NUC, nuclear fraction, Control values can be taken from Tables 1 and 2.

The section thickness was comparable with the diameter of most of the microsomal vesicles; thus the number of ferritin images per unit membrane area, calculated from the electron micrographs, is certainly underestimated, particularly if the smaller vesicles are considered. However, this underestimation is probably not sufficient to account for the low extent of labelling observed, since most of the microsomal vesicles were found in perimeter classes 3–5, in which the average ferritin load was proportional to perimeter length. Therefore the observed ferritin labelling must have been influenced by other factors and not solely by the geometry of the sections.

Several factors could contribute to the low extent of binding. (1) Bound ferritin conjugates may dissociate from the microsomal vesicles during separation from unbound conjugates (Remacle *et al.*, 1976). (2) Steric hindrance as a result of enzyme clustering may prevent recognition of some enzyme molecules (Remacle *et al.*, 1976). (3) The distribution of the enzyme within the membrane may be such that only a fraction of the molecules are accessible at one particular time. Incubations were performed at 4°C. At this temperature microsomal vesicles may be quite rigid (Eletr *et al.*, 1973; Mabrey *et al.*, 1977), and the low extent of binding may reflect the fraction of enzyme molecules that were accessible at the time the membranes were chilled. Thus incubation at higher temperatures may result in more binding, since enzyme molecules would be trapped as they became accessible by diffusion within the membrane.

The quantitative analysis of the different fractions of the endoplasmic reticulum (unfractionated microsomal preparation and smooth and rough microsomal fractions) showed that the extent of labelling increased in proportion to the perimeter length of the vesicles, except for the smooth microsomal fraction, with which the ferritin load to the largest vesicles was less than expected. This could result from a greater contamination of this fraction with Golgi elements and plasma-membrane fragments (Autuori *et al.*, 1975). Otherwise, it would appear that epoxide hydrolase molecules are evenly distributed throughout the different perimeter classes from each of the microsomal fractions.

The nuclear outer membrane showed a ferritin load that was very similar to that of the microsomal fraction. This finding, which clearly demonstrates association of epoxide hydrolase with the nuclear membrane, is at first sight somewhat surprising, since the specific epoxide hydrolase activity of the nuclear fraction was much lower than that of the microsomal fraction (Table 1), and was comparable with that reported by other laboratories (Jernström *et al.*, 1976; Bornstein *et al.*, 1978; Mukhtar *et al.*, 1979; Stasiecki *et al.*, 1980). However, the contribution of the nuclear membrane to the total nuclear protein is small (Agutter & Gleed, 1980), and the specific epoxide hydrolase activity reported for isolated nuclear envelope (Fahl *et al.*, 1978) was similar to that of microsomal fractions. Moreover, Matsuura *et al.* (1978) reported that nuclear outer membranes and microsomal vesicles were labelled to similar extents by ferritin conjugates of antibodies to cytochrome *P*-450. With the mitochondrial, lysosomal and peroxisomal fractions very little difference in the degree of binding was observed, whether incubations were performed with specific or non-specific conjugates. The lack of specific binding to peroxisomes is of interest in view of the hypothesis that these organelles are derived from locally differentiated zones of endoplasmic reticulum (Novikoff & Shin, 1964; Tsukada *et al.*, 1966; Hruban *et al.*, 1974).

Treatment of animals with phenobarbitone and 2-acetamidofluorene resulted in the expected increases of epoxide hydrolase activity in microsomal fractions (Oesch *et al.*, 1973; Levin *et al.*, 1978). Acetamidofluorene also induced the enzyme in

nuclear preparations. The specific binding to all microsomal preparations (unfractionated microsomal preparation and rough and smooth microsomal fractions) increased in accordance with the enzyme activities (Fig. 5). The most likely interpretation of these findings is that the elevated activity results from an increased number of enzyme molecules within the membrane. However, it is conceivable that the accessibility of the enzyme is influenced by treatment-related changes in membrane composition (Davison & Wills, 1974). The specific binding to nuclear membranes was increased to a greater extent than was nuclear enzyme activity after acetamidofluorene treatment. The reason for this is not known.

In conclusion, the present study demonstrates that epoxide hydrolase is accessible from the cytoplasmic surface of microsomal and nuclear membranes. The numbers of accessible enzyme molecules per unit membrane area were very similar in these membrane fractions, and were increased in accordance with the enzyme activity when rats were treated with known epoxide hydrolase inducers. Specific antibody binding to intact peroxisomes, lysosomes and mitochondria could not be detected, which implies that the outer surface membranes of these organelles do not contain epoxide hydrolase. Binding to microsomal fractions gave no indication that the enzyme may be inhomogeneously distributed throughout the endoplasmic reticulum.

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