Biochem. J. (1980) **190**, 737–746
Printed in Great Britain

Asymmetric distribution of cytochrome *P*-450 and NADPH–cytochrome *P*-450 (cytochrome *c*) reductase in vesicles from smooth endoplasmic reticulum of rat liver

Michael B. COOPER, John A. CRAFT,* Margaret R. ESTALL and Brian R. RABIN
Department of Biochemistry, University College London, Gower Street, London WC1E 6BT, U.K.

(*Received 17 January 1980*)

1. The topography of cytochrome *P*-450 in vesicles from smooth endoplasmic reticulum of rat liver has been examined. Approx. 50% of the cytochrome is directly accessible to the action of trypsin in intact vesicles whereas the remainder is inaccessible and partitioned between luminal-facing or phospholipid-embedded loci. Analysis by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis reveals three major species of the cytochrome. Of these, the variant with a mol.wt. of 52000 is induced by phenobarbitone and this species is susceptible to trypsin. 2. After trypsin treatment of smooth membrane, some NADPH–cytochrome *P*-450 (cytochrome *c*) reductase activity remains and this remaining activity is enhanced by treatment with 0.05% deoxycholate, which renders the membranes permeable to macromolecules. In non-trypsin-treated control membranes the reductase activity is increased to a similar extent. These observations suggest an asymmetric distribution of NADPH–cytochrome *P*-450 (cytochrome *c*) reductase in the membrane. 3. As compared with dithionite, NADPH reduces only 44% of the cytochrome *P*-450 present in intact membranes. After tryptic digestion, none of the remaining cytochrome *P*-450 is reducible by NADPH. 4. In the presence of both a superoxide-generating system (xanthine plus xanthine oxidase) and NADPH, all the cytochrome *P*-450 in intact membrane (as judged by dithionite reducibility) is reduced. The cytochrome *P*-450 remaining after trypsin treatment of smooth vesicles cannot be reduced by this method. 5. The superoxide-dependent reduction of cytochrome *P*-450 is prevented by treatment of the membranes with mersalyl, which inhibits NADPH–cytochrome *P*-450 (cytochrome *c*) reductase. Thus the effect of superoxide may involve NADPH–cytochrome *P*-450 reductase and cytosolically orientated membrane factor(s).

The topographical distribution of the intrinsic membrane proteins of the endoplasmic reticulum can be investigated by the use of proteolytic enzymes (Nilsson & Dallner, 1977). Since these enzymes cannot penetrate the intact phospholipid bilayer (Ito & Sato, 1969; Nilsson & Dallner, 1977; Nilsson *et al.*, 1978; Craft *et al.*, 1979*a*), only those proteins with susceptible bonds exposed on the outer cytosolic face of the vesicles are subject to degradation. However, vesicles can be made permeable to proteinases by treatment with low concentrations of deoxycholate (Kreibich *et al.*, 1973) or by disruption by sonication. In this way it has been shown that the enzymatic activities associated with cytochrome b_5 and NADPH–cytochrome *c* reductase are located on the cytosolic face of the membranes, whereas glucose-6-phosphatase is positioned on the inner luminal face (Nilsson & Dallner, 1977).

The electron-transport chain of the mixed function oxygenase of the endoplasmic reticulum is composed of the flavoprotein, NADPH–cytochrome *P*-450 (cytochrome *c*) reductase (EC 1.6.2.4), and cytochrome *P*-450. Experiments involving reconstitution of the system from isolated components have shown that electron transfer requires the presence of phospholipids (Strobel *et al.*, 1970). The mixed-function oxygenase is enzymatically active towards a wide range of xenobiotics and naturally

* Present address: Department of Biology, Glasgow College of Technology, Cowcaddens Road, Glasgow, Scotland, U.K.

0306-3283/80/090737-10$01.50/1

occurring compounds such as steroids (Kuntzman, 1969; Gillette *et al.*, 1972).

The reductase enzyme can utilize cytochrome *c* as terminal electron acceptor and is often referred to as NADPH–cytochrome *c* reductase (Phillips & Langdon, 1962). It has been demonstrated that cytochrome *P*-450, NADPH–cytochrome *c* reductase and the drug-metabolizing activities of microsomes are induced by the administration of substances such as phenobarbitone to animals (Ernster & Orrenius, 1965; Rees, 1979). In addition, immunochemical studies have shown that antibodies raised to purified NADPH–cytochrome *c* reductase are effective inhibitors of microsomal NADPH-linked drug hydroxylations (Masters *et al.*, 1971). It now seems clear from studies with the reconstituted electron transport chain with purified cytochrome *P*-450 and NADPH–cytochrome *c* reductase that cytochrome *P*-450 is the natural electron acceptor for the NADPH–cytochrome *c* reductase (Lu *et al.*, 1969; Strobel & Coon, 1971; Vermilion *et al.*, 1974). Cytochrome *P*-450 exists in multiple forms differing in molecular weight and substrate specificity (Haugen *et al.*, 1975; Bergman & Dallner, 1976; Coon *et al.*, 1976; Haugen & Coon, 1976).

We have investigated the distribution of both the cytochrome and its associated reductase in vesicles from smooth endoplasmic reticulum by digestion with trypsin, which degrades accessible cytochrome *P*-450 to a catalytically inactive form that is spectrally distinguishable as cytochrome *P*-420.

Materials and methods

Animals

Male Sprague–Dawley rats weighing 200g and permitted food and water *ad libitum* were used in this study. Sodium phenobarbitone in aqueous 0.9% NaCl was administered as five successive daily single intraperitoneal injections each of 40mg/kg body wt., before killing the animals on the sixth day. Control animals received an identical injection protocol with an equivalent volume of saline.

Preparation of smooth endoplasmic reticulum and trypsin treatment

Vesicles from smooth endoplasmic reticulum were prepared from the liver as previously described (Blyth *et al.*, 1971). Digestion with trypsin was carried out by incubating membrane (2mg of protein/ml) with trypsin (50μg/mg of membrane protein) in a sucrose-containing buffer at 30°C for 45min or the time indicated. The buffer (pH7.5 at 20°C) consisted of sucrose (0.25M), $MgCl_2$ (5mM), KCl (25mM), EDTA (1mM) and Tris (50mM). The reaction was terminated by the addition of soya bean trypsin inhibitor (2mg/mg of trypsin) and was cooled on ice. Control membranes were similarly incubated, but in the absence of trypsin. The incubated membranes were centrifuged at 105000$g_{av.}$ at 4°C for 1h to separate the membranes from any released fragments of proteins, some of which can remain catalytically active [for example NADPH–cytochrome *P*-450 (cytochrome *c*) reductase; Masters *et al.* (1975)]. The membrane pellet was resuspended in a solution containing $MgCl_2$ (5mM), KCl (50mM) and Tris (50mM), pH7.5 at 20°C, and the suspension was assayed for the various enzymes detailed in the text.

Enzyme assays

Cytochrome *P*-450 content was determined by the spectral method of Omura & Sato (1964). Approx. 1mg of membrane protein in 1ml of a solution containing sucrose (0.25M), $MgCl_2$ (5mM), KCl (25mM), EDTA (1mM) and Tris (50mM), pH7.5 at 20°C, was placed into each of two 1ml glass spectrophotometer cuvettes. The contents of one cuvette were saturated with CO and a baseline spectrum was drawn from 400 to 500nm. A few crystals of sodium dithionite were then added to each cuvette and dissolved, and the spectrum was rescanned. The concentration of cytochrome *P*-450 present was calculated, assuming the difference $\varepsilon_{450}-\varepsilon_{490}$ to be $91\,mM^{-1}\cdot cm^{-1}$. Measurements were made with a Unicam SP.8000 spectrophotometer by using the sample compartment close to the photomultiplier. Alternatively, the integrity of the electron transport chain was examined by reduction of cytochrome *P*-450 with NADPH (Orrenius *et al.*, 1969). This was carried out in the same manner as the dithionite reduction of cytochrome *P*-450 with the exception that NADPH was added to both cuvettes to a final concentration of 0.1mM in place of the sodium dithionite.

NADPH–cytochrome *P*-450 (cytochrome *c*) reductase was assayed by the method of Phillips & Langdon (1962) with cytochrome *c* as terminal electron acceptor. Incubations (1ml) were carried out in glass cuvettes at 30°C in a medium (pH7.5) containing sodium phosphate (0.1M), EDTA (1mM), cytochrome *c* (40μM), KCN (0.5mM) and 10–20μg of microsomal protein. The reaction was initiated by the addition of NADPH to a final concentration of 0.1mM. The reduction of cytochrome *c* was followed at 550nm and under these conditions the progress curves were linear for up to 5min.

NADH–cytochrome *c* reductase (EC 1.6.99.3) was assayed by the method of Dallner *et al.* (1966). Glucose-6-phosphatase (EC 3.1.3.9) was determined by the method of Leskes *et al.* (1971). Xanthine oxidase (EC 1.2.3.2) was assayed by the method of Fridovich (1962). Superoxide production was monitored by the oxidation of adrenalin to adrenochrome at pH7.5 (Mazur *et al.*, 1956; Aust *et al.*, 1972).

General methods and chemicals

Protein was determined by the method of Lowry *et al.* (1951). Phospholipid was extracted by the method of Folch *et al.* (1957) and its phosphorus content was determined as described by Chen *et al.* (1956). Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis was carried out by the method of Laemmli (1970).

Trypsin, twice crystallized from bovine pancreas (Sigma type I), soya bean trypsin inhibitor (Sigma type 1-S), xanthine, xanthine oxidase (Sigma grade I) and superoxide dismutase (EC 1.15.1.1) (Sigma type I) were obtained from the Sigma Chemical Co., Poole, Dorset, U.K. All other chemicals were of A.R. grade or equivalent.

Results

Effects of trypsin on enzyme activities of the smooth endoplasmic reticulum in non-induced rats

When vesicles from smooth membrane are incubated with trypsin, their appearance in the electron microscope is unchanged except for a small apparent decrease in size. Trypsin treatment degrades only those proteins on the outer cytosolic face of the membranes, in the absence of detergent. The luminally facing protein glucose-6-phosphatase is unaffected (Fig. 1). The phospholipid bilayer is thus maintained as a permeability barrier to macromolecules. The content of cytochrome *P*-450, as detectable spectrally by reduction with dithionite in the presence of CO, decreases to approx. 50% of that of the control membranes (Fig. 1) on treatment with trypsin. Proteinase denaturation of cytochrome *P*-450 is characterized by a decrease in the absorption of the CO complex at 450nm with a stoichiometric and concomitant increase in absorption of the enzymically inactive cytochrome at 420nm. The effect of the proteinase is thus not to lower the absorption coefficient of the cytochrome. In the presence of 0.05% sodium deoxycholate, which renders the phospholipid bilayer permeable to macromolecules without releasing membrane components (Kreibich *et al.*, 1973), the spectrally detectable cytochrome *P*-450 content is further decreased to approx. 20% of its initial value. This further decrease is not observed if soya bean trypsin inhibitor is added to the incubation before the addition of deoxycholate. Fig. 1 shows that the activity of glucose-6-phosphatase in intact vesicles is unchanged by treatment with trypsin. The addition of 0.05% deoxycholate, however, results in the loss of all glucose-6-phosphatase activity. In contrast, NADH–cytochrome *c* reductase activity is totally abolished by treatment of membrane vesicles with trypsin, in the absence of deoxycholate.

The use of detergent to investigate the location of proteins is, to some extent, ambiguous since effects additional to rendering the phospholipid bilayer permeable to macromolecules could be involved.

To examine this possibility, smooth endoplasmic reticulum was incubated with trypsin, as described,

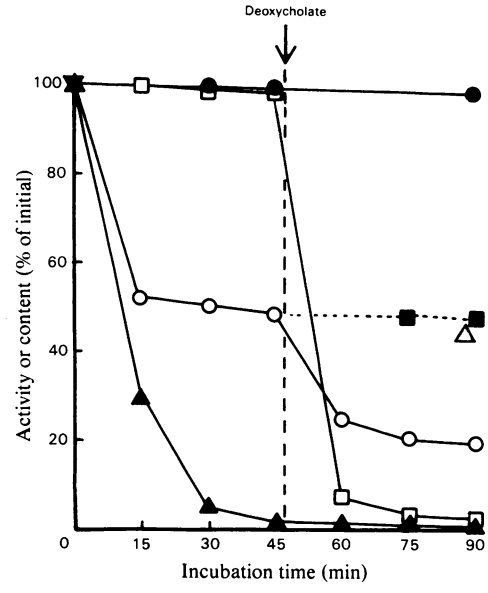

Fig. 1. *Effects of trypsin in the presence and absence of sodium deoxycholate on the activities of cytochrome P-450, glucose-6-phosphatase and NADH–cytochrome c reductase of the smooth endoplasmic reticulum in non-induced rats*

Vesicles from smooth endoplasmic reticulum at a protein concentration of 1.8mg/ml were incubated with trypsin (50μg/mg of membrane protein) or alone (controls) as detailed in the Materials and methods section. After 45min incubation, sodium deoxycholate was added to a final concentration of 0.05%. Aliquots were withdrawn at the time intervals indicated, soya bean trypsin inhibitor (2mg/mg of trypsin) was added and assays were performed for the above enzymes. ★, Zero-time level for all assays (=100%); ●, cytochrome *P*-450 content of incubation without trypsin (control); ○, cytochrome *P*-450 content of incubation with trypsin; △, cytochrome *P*-450 content of incubation with trypsin but without deoxycholate; ■, cytochrome *P*-450 content of incubation with trypsin; at 45min soya bean trypsin inhibitor (2mg/mg of trypsin) was added immediately before the addition of deoxycholate; □, glucose-6-phosphatase activity of incubation with trypsin; ▲, NADH–cytochrome *c* reductase activity of incubation with trypsin. The Figure contains the average of data from four separate experiments. In no case did the individual value obtained vary by more than ±5% of that given. Cytochrome *P*-450 was determined by dithionite reduction. A cytochrome *P*-450 content of 100% represents 68pmol of cytochrome *P*-450/μg of lipid P.

until the cytochrome *P*-450 content was lowered to a constant level. The membranes were then disrupted by sonication for 1 min at 0°C to introduce the trypsin into the lumen of the vesicles. As shown in Fig. 2 the effects of sonication are identical with those produced by treatment with deoxycholate.

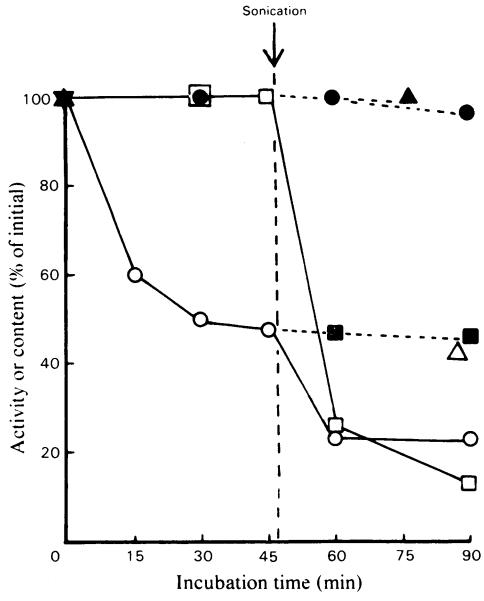

Fig. 2. *Effects of trypsin treatment on the activities of cytochrome P-450 and glucose-6-phosphatase of the smooth endoplasmic reticulum before and after ultrasonication treatment in non-induced rats*

Vesicles from smooth endoplasmic reticulum were incubated with trypsin or alone (controls) as described in the Materials and methods section. After 45 min the incubation mixtures were ultrasonicated for 1 min at 0°C and the reaction was allowed to proceed. Aliquots were withdrawn, soya bean trypsin inhibitor (2 mg/mg of trypsin) was added and assays were performed for cytochrome *P*-450 and glucose-6-phosphatase. ★, Zero-time level for all assays (=100%); ●, cytochrome *P*-450 content of incubation without trypsin; ○, cytochrome *P*-450 content of incubation with trypsin; △, cytochrome *P*-450 content of incubation with trypsin, but without any addition of deoxycholate; ■, cytochrome *P*-450 incubated with trypsin, at 45 min trypsin inhibitor (2 mg/mg of trypsin) was added immediately before ultrasonication; □, glucose-6-phosphatase activity of incubation with trypsin; ▲, glucose-6-phosphatase activities in the presence of trypsin, at 45 min trypsin inhibitor (2 mg/mg of trypsin) was added immediately before ultrasonication. The Figure contains the average of data from four separate experiments. In no case did the individual value obtained vary by more than ±5% of that given. Cytochrome *P*-450 was determined by the dithionite reduction method. A cytochrome *P*-450 content of 100% represents 68 pmol/μg of lipid P.

Membrane locus of phenobarbitone-induced cytochrome P-450

The distribution of the cytochrome *P*-450 variant induced by sodium phenobarbitone was examined by its susceptibility to trypsin digestion as described above. The results are shown in Table 1. In contrast with control animals, approx. 80% of the cytochrome *P*-450 is lost by the trypsin treatment. The amount of cytochrome *P*-450 remaining after trypsin treatment is similar in control and phenobarbitone-treated animals. It seems, therefore, that the cytochrome *P*-450 in the induced animals is located on the cytosolic face of the smooth membranes.

Fig. 3 shows sodium dodecyl sulphate/polyacrylamide-gel electrophoretograms of membrane proteins from control and sodium phenobarbitone-treated animals before and after trypsin digestion. In the smooth endoplasmic reticulum of control animals three major cytochrome *P*-450 bands can be observed of mol.wts. 52000, 49000 and 47000 respectively. These bands were characterized as haemoproteins by staining for residual peroxidase activity (Welton & Aust, 1974) and by comparison of the bands with those of highly purified preparation of cytochrome *P*-450 (Craft *et al.*, 1978, 1979*b*). After trypsin treatment, the variant of mol.wt. 49000 is almost totally abolished and that of mol.wt. 52000 is present in decreased amount. Membranes from sodium phenobarbitone-treated animals appear to contain the same three cytochrome *P*-450 variants as are present in the control membranes, but in stoichiometrically different amounts. The species of mol.wt. 52000 is present in increased quantities and after trypsin treatment is greatly diminished. These observations suggest that the phenobarbitone-induced cytochrome *P*-450 variant is sited at a locus on the cytosolic face of the reticulum, and is of mol.wt. 52000. The possibility that other phenobarbitone-inducible proteins, with the same electrophoretic mobility as the cytochrome *P*-450 species, are present on the gels cannot be conclusively eliminated.

Asymmetric distribution of NADPH–cytochrome P-450 (cytochrome c) reductase in the transverse plane of the membrane in non-induced rats

NADPH–cytochrome *P*-450 (cytochrome *c*) reductase has been reported to be located on the cytosolic face of the membrane (Orrenius *et al.*, 1969; Nilsson & Dallner, 1977). Trypsin treatment of the membranes releases a fragment that has catalytic activity for the oxidation of NADPH but cannot participate in the transfer of electrons to cytochrome *P*-450 (Masters *et al.*, 1975). However, as shown in Fig. 4, when the membrane fraction is separated from the tryptic fragments, some reduct-

Table 1. *Cytochrome P-450 contents of vesicles from smooth membranes from control and phenobarbitone-treated rats were incubated in the presence of trypsin and deoxycholate*

Vesicles from smooth membranes were prepared from phenobarbitone-treated and control animals and cytochrome *P*-450 was determined by dithionite reduction as detailed in the Materials and methods section. Membrane was incubated with trypsin under the conditions described in that section. After 45 min of incubation, an aliquot was withdrawn, treated with soya bean trypsin inhibitor (2 mg/mg of trypsin) and its cytochrome *P*-450 content was determined. Deoxycholate was then added to the incubations to a final concentration of 0.05% and, after 45 min further incubation, aliquots were withdrawn and treated as previously. The results show the means (±S.D.) from four separate experiments.

Treatment	Rats ...	Cytochrome *P*-450 content (pmol/μg of lipid P) in smooth membrane	
		Control	Phenobarbitone-treated
None		67 ± 10	109 ± 14
Trypsin (45 min)		33 ± 7	24 ± 8
Trypsin (45 min) followed by 0.05% deoxycholate for 45 min		17 ± 5	19 ± 6

ase activity can still be detected. This residual activity is greatly enhanced when the membrane vesicles are made permeable to macromolecules, such as cytochrome *c*, by treatment with deoxycholate. Similarly, if non-trypsin-treated membrane is subjected to deoxycholate treatment the activity of the NADPH–cytochrome *c* reductase is also increased (Fig. 4).

Effects of trypsin on the microsomal mixed-function oxygenase electron-transport chain

Cytochrome *P*-450 can be reduced chemically by sodium dithionite (Omura & Sato, 1964) or enzymatically by NADPH–cytochrome *P*-450 (cytochrome *c*) reductase in the presence of NADPH (Mason *et al.*, 1965; Orrenius *et al.*, 1969) to give the form that binds CO with an absorption peak at 450 nm. As shown in Table 2, more than twice as much spectrally detectable cytochrome *P*-450 is produced by chemical reduction by dithionite as compared with enzymatic reduction by NADPH. After trypsin treatment of the vesicles there is no spectrally detectable cytochrome *P*-450 produced on reduction with NADPH and the remaining cytochrome is detectable only by dithionite treatment.

There is clearly a fraction of the cytochrome *P*-450 that is not directly reducible by NADPH. This fraction of the protein may be located on the luminal face of the membrane or it may be located in such a manner that it is unable to interact with NADPH–cytochrome *P*-450 reductase. In the light of the data in Figs. 1 and 2 and Table 2 we favour the former explanation.

Attempts to reduce the putative luminal cytochrome *P*-450 by NADPH in the presence of 0.05% sodium deoxycholate were unsuccessful. It is, however, possible that the detergent, by interaction with the membrane phospholipids, disrupts the coupling of NADPH–cytochrome *P*-450 reductase and cytochrome *P*-450. Ultrasonication treatment should introduce NADPH to the inside of the vesicles. However, this procedure caused very little reduction of the putative, cisternally located cytochrome *P*-450 (results not shown). The reason may be that only short exposure (1 min) to ultrasonics is possible because extended treatment causes conversion of cytochrome *P*-450 into the denatured *P*-420 form.

Involvement of superoxide in the reduction of cytochrome P-450

It has been reported that, in a reconstituted cytochrome *P*-450 hydroxylation system, superoxide may be involved in the metabolism of benzphetamine and ethylmorphine (Strobel & Coon, 1971). Superoxide can be generated conveniently by the reaction between xanthine and xanthine oxidase (Fridovich & Handler, 1958; Strobel & Coon, 1971). We have investigated the effects of superoxide on the enzymic reduction of cytochrome *P*-450. In Fig. 5 it can be seen that, after reduction of cytochrome *P*-450 with NADPH, the addition of a superoxide-generating system causes the appearance of a further increment of cytochrome *P*-450. The amount of cytochrome *P*-450 measurable by treatment with NADPH and a superoxide-generating system is the same as that produced by dithionite reduction. In the absence of NADPH, but in the presence of the xanthine/xanthine oxidase superoxide-generating system, no reduction at all of cytochrome *P*-450 could be detected.

The reduction observed could have been due to a factor derived from either the substrate, enzyme or the other products of the superoxide-generating system rather than superoxide *per se*. These possi-

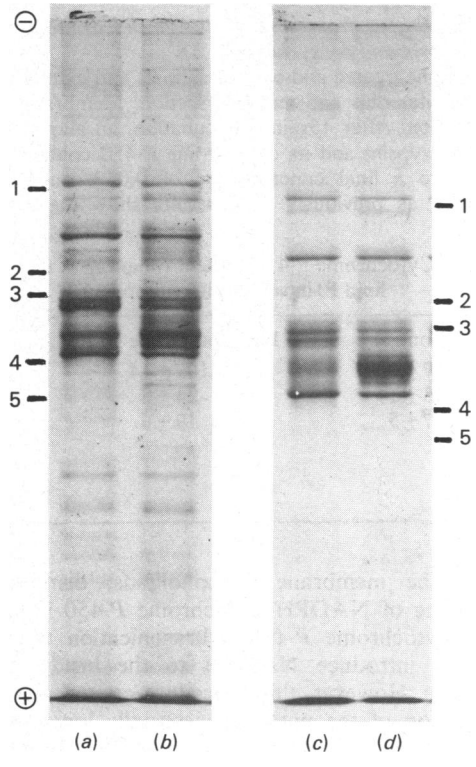

Fig. 3. *Electrophoretic separation of membrane proteins from membranes from control and phenobarbitone-induced rats, both before and after trypsin digestion of the cytosolic-facing proteins*

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis was carried out by the method of Laemmli (1970), with a 3% (w/v) acrylamide stacking gel, pH 6.8, and 10% (w/v) acrylamide separation gel, pH 8.8. Samples were run into the gels for 1 h at 10 mA/gel and then were electrophoresed for 4 h at 30 mA/gel, constant current. Each sample is derived from a quantity of membrane containing 0.6 μg of lipid P. Membranes were treated with trypsin as described in the Materials and methods section. (*a*) Control smooth membrane treated with trypsin; (*b*) control smooth membranes not treated with trypsin; (*c*) phenobarbitone-induced smooth membrane treated with trypsin: (*d*) phenobarbitone-induced smooth membrane not treated with trypsin. The gels were calibrated for molecular weight by electrophoresis, on the same slab gel, of a mixture of purified proteins which migrated to the positions indicated: 1, phosphorylase A (mol.wt. 94000); 2, bovine serum albumin (mol.wt. 66000); 3, pyruvate kinase (mol.wt. 57000); 4, ovalbumin (mol.wt. 44000); 5, alcohol dehydrogenase (mol.wt. 41000).

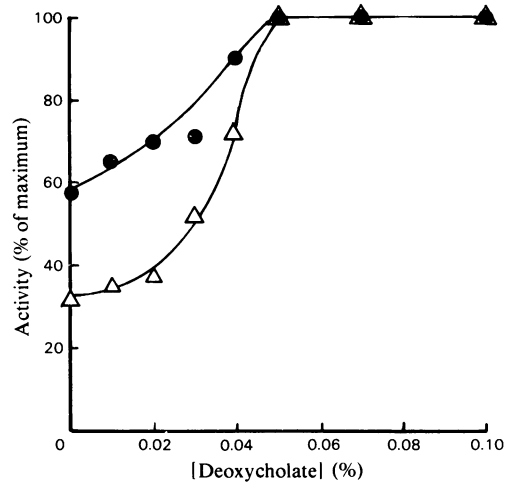

Fig. 4. *NADPH–cytochrome P-450 (cytochrome c) reductase activity of control and trypsin-treated smooth membrane vesicles, in the presence of sodium deoxycholate, from non-induced rats*

NADPH–cytochrome *P*-450 (cytochrome *c*) reductase content of trypsin-treated and control membranes was assayed in the presence of sodium deoxycholate at the concentrations indicated, as described in the Materials and methods section. ●, Control smooth membrane vesicles; △, trypsin-treated smooth membrane vesicles. The smooth membrane vesicles were treated with trypsin for 45 min as described in the Materials and methods section and the digestion was terminated by addition of soya bean trypsin inhibitor (2 mg/mg of trypsin). The results are presented as a percentage of the maximum activity expressed under these conditions. Maximum activity was 100 nmol of cytochrome *c* reduced/h per μg of lipid P for control membranes and 32 nmol of cytochrome *c* reduced/h per μg of lipid P for trypsin-treated membranes.

bilities were eliminated by a series of control experiments illustrated in Table 3. The extra increment of cytochrome *P*-450 above that produced by NADPH was obtained only with the complete xanthine/xanthine oxidase system. It was not produced by the reaction products, H_2O_2 and uric acid, and its production was inhibited by superoxide dismutase. These observations strongly suggest that the superoxide ion is the active species involved in this further reduction process. However, although the above data clearly implicate the superoxide ion in the reduction of cytochrome *P*-450, any involvement of NADPH–cytochrome *P*-450 reductase in the process is conjectural. The organic mercurial, mersalyl, is a potent inhibitor of NADPH–cytochrome *P*-450 reductase (Franklin & Estabrook, 1971). At a concentration of mersalyl of 60 μM, superoxide production was not significantly affected, whereas NADPH–cytochrome *P*-450 (cytochrome *c*) reductase activity was totally inhibited (results not shown). To investigate the involvement of NADPH–cytochrome *P*-450 (cytochrome *c*) reductase in the superoxide phenomenon,

Table 2. *Reduction of cytochrome P-450 in control and trypsin-treated smooth membrane vesicles from non-induced rats*

Trypsin treatment of smooth vesicles was carried out as detailed in the Materials and methods section. Control membranes were similarly incubated, but in the absence of trypsin. The CO-binding spectrum of cytochrome *P*-450 was determined by the method of Omura & Sato (1964) as described in the Materials and methods section. Results show the means (±S.D.) obtained from four separate experiments. Abbreviation: n.d., not detectable.

Reductant	Membrane ...	Cytochrome *P*-450 reduced (pmol/μg of lipid P)	
		Control	Trypsin-treated
NADPH			
0.2 mM		28.2 ± 8.0	n.d.
1.0 mM		28.2 ± 8.0	n.d.
Sodium dithionite		63.0 ± 11.0	28.0 ± 5.0

Table 3. *Reduction of cytochrome P-450, additional to that produced by NADPH alone, by a superoxide-generating system and by the components and products of that system*

The smooth membrane vesicles were prepared from non-induced rats. The CO-binding spectrum of cytochrome *P*-450 was determined as described in the Materials and methods section. After addition of NADPH to the cuvettes, the component or product indicated was added, at the concentrations indicated, to both cuvettes. After 5 min incubation, or when any increase in absorbance at 450 nm ceased to occur, the spectrum was scanned again. Results are the means (±S.D.) from four separate experiments. Abbreviation: n.d., not detectable.

Addition	Membrane ...	Cytochrome *P*-450 reduced additional to that reduced by NADPH alone (pmol/μg of lipid P)	
		Control	Trypsin-treated
Xanthine (2 mM) + xanthine oxidase (0.1 mg/ml)		33.7 ± 7.0	n.d.
Xanthine (2 mM) + xanthine oxidase (0.1 mg/ml) + superoxide dismutase (0.5 mg/mi)		<0.5	n.d.
Xanthine (2 mM)		n.d.	n.d.
Xanthine oxidase (0.1 mg/ml)		n.d.	n.d.
Uric acid (2 mM)		n.d.	n.d.
H_2O_2 (2 mM)		n.d.	n.d.
Sodium dithionite (few crystals)		34.8 ± 7.0	18.0 ± 5.0

Table 4. *Effects of mersalyl on the reduction of cytochrome P-450 by NADPH and a superoxide-generating system in vesicles from smooth membrane of non-induced rats*

Cytochrome *P*-450 was assayed as described in the Materials and methods section. Where indicated, mersalyl was included to a final concentration of 60 μM in each pair of cuvettes before saturation of the measuring cell by CO. Other additions were made to both measuring and reference cells at the final concentration indicated. Smooth endoplasmic reticulum was incubated with trypsin as described in the Materials and methods section. The results show the means (±S.D.) from four separate experiments. Abbreviation: n.d., not detectable.

Addition	Membrane ...	Cytochrome *P*-450 reduced (pmol/μg of lipid P)		
		Control	Control + mersalyl	Trypsin-treated
NADPH (0.2 mM)		28.0 ± 7.0	n.d.	n.d.
NADPH (0.2 mM) + xanthine (2 mM) + xanthine oxidase (0.1 mg/ml)		64.2 ± 10.0	n.d.	n.d.
Sodium dithionite (few crystals)		64.8 ± 10.0	50.0 ± 6.0	28.0 ± 5.0

the effects of mersalyl were determined and the results are summarized in Table 4. Mersalyl completely blocks the reduction of cytochrome *P*-450 by NADPH, alone or in the presence of a superoxide-generating system. In the presence of mersalyl, cytochrome *P*-450 can be detected only by reduction with sodium dithionite. The fact that the majority of the cytochrome *P*-450 present can still

be detected in the presence of mersalyl by dithionite reduction indicates that the cytochrome is itself unaffected by the presence of the reductase inhibitor. It is, however, a possibility that mersalyl in some way affects subtle functional characteristics of cytochrome *P*-450 without altering its absorption spectrum.

Effects of trypsin on the superoxide-induced reduction of cytochrome P-450

After trypsin treatment of smooth endoplasmic reticulum as outlined in the Materials and methods section, no cytochrome *P*-450 could be detected by reduction with NADPH. It was of interest to determine whether reduction could be accomplished by NADPH in the presence of superoxide. As shown in Table 4, under conditions where superoxide totally reduced cytochrome *P*-450 in control membranes, no reduction of the cytochrome in trypsin-treated membranes was observed. This would suggest the involvement in the reduction of cisternally located cytochrome *P*-450 of a trypsin-sensitive factor on the cytosolic face of the vesicles.

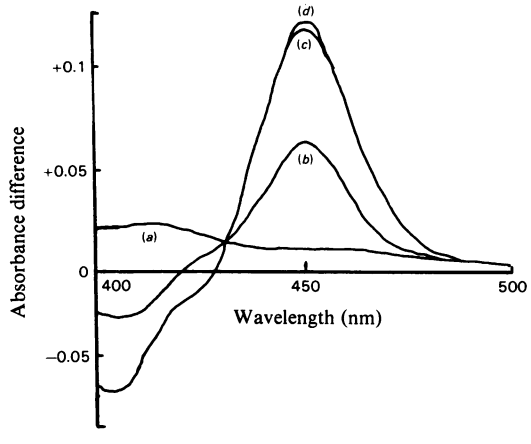

Fig. 5. *Reduction of cytochrome P-450 in smooth membrane vesicles from non-induced rats by NADPH and by a superoxide-generating system*

The spectrum of the CO complex of reduced cytochrome *P*-450 was determined with NADPH as reductant as described in the Materials and methods section. Further additions of NADPH caused no extra reduction. Xanthine oxidase (0.1 mg) was then added to each cuvette followed by the addition of xanthine, to a final concentration of 2 mM. After an incubation period of 5 min at 25°C, or when the absorbance at 450 nm had ceased to increase, the spectrum was again scanned. A few crystals of sodium dithionite were then added to each cuvette to determine the total cytochrome *P*-450 content. (*a*) Baseline spectrum; (*b*) CO-binding spectrum after reduction with NADPH; (*c*) CO-binding spectrum after reduction with the superoxide-generating system; (*d*) CO-binding spectrum after reduction with sodium dithionite.

Discussion

Membrane proteins are asymmetrically distributed in the transverse plane of the phospholipid bilayer, as indicated by their varying susceptibilities to enzymatic proteolysis. The topology of xenobiotically induced enzymes in smooth endoplasmic reticulum is of great interest due to the extensive proliferation that this organelle exhibits on induction. Our data confirm the previous report (Nilsson & Dallner, 1977) that, as in total microsomes, glucose-6-phosphatase is sited at a locus on the inner luminal (cisternal) face of vesicles from the endoplasmic reticulum. In contrast, NADH–cytochrome *c* reductase is confirmed to be located on the outer cytosolic face of the reticulum (Takesue & Omura, 1968; Ito, 1973). The distribution of cytochrome *P*-450 has been examined (Nilsson & Dallner, 1977; Nilsson *et al.*, 1978) and the reported trypsin resistance of a species of cytochrome *P*-450 has been used as the basis of a method for its purification (Welton *et al.*, 1975). The results presented in the present paper suggest the existence of two, and possibly three, different loci for cytochrome *P*-450 in reticular membranes. The first locus is the exposed cytosolic face of the membranes. Amongst the variants of the cytochrome so located are those of mol.wts. 49000 and 52000, the latter being the variant induced by phenobarbitone. The second locus for the cytochrome is on the luminal face of the endoplasmic reticulum. The variants present in this location are not induced by phenobarbitone. Some molecules of cytochrome *P*-450 can still be detected when proteins on both the luminal and cytosolic faces of membrane vesicles are digested by trypsin. This suggests that a third possible location for the cytochrome, embedded in the phospholipid bilayer, may exist. However, it is equally possible that one of the variants of cytochrome *P*-450 on either side of the membrane may have no bonds exposed that are susceptible to tryptic digestion.

When trypsin is introduced to the vesicle lumen by ultrasonication in the absence of detergent, the same pattern of cytochrome *P*-450 degradation is observed as in the presence of sodium deoxycholate. It would thus seem unlikely that the effect of sodium deoxycholate is to make available sites for the action of trypsin on cytochrome *P*-450 molecules on the cytosolic face of the reticulum that are ordinarily refractory to trypsin. A report (Nilsson *et al.*, 1978) has suggested that cytochrome *P*-450 is solely located on the cytosolic face of the microsomes. By performing the digestion of proteins of the cytosolic face of total microsomes with trypsin plus a

non-specific bacterial proteinase it is claimed that destruction of 90% of the cytochrome is achieved. When either enzyme alone is used, only 50% of the cytochrome is susceptible to proteolysis. Confirmation of their observation was suggested by the fact that an agent capable of inactivating cytochrome *P*-450, but not of traversing the phospholipid bilayer, also caused the loss of the majority of the cytochrome. However, it is difficult to explain why a totally non-specific proteinase should behave in a different fashion when used in the presence of trypsin. The possibility of damage to the phospholipid bilayer by processes such as accelerated lipid peroxidation or phospholipase activation cannot be ruled out. Such damage could render the microsomal vesicles leaky and permeable to macromolecules. Indeed this is suggested by the fact that approx. 20% of the cisternally located glucose-6-phosphatase is labile to their treatments. The different metabolic state of the animals used, and the fact that we have used smooth endoplasmic reticulum rather than total microsomes, might also have a bearing on the discrepancy between our conclusions and those of Nilsson *et al.* (1978).

The finding that the cytochrome *P*-450 variant induced by sodium phenobarbitone resides on the cytosolic face of the reticulum is at variance with a report (Welton *et al.*, 1975) claiming the purification of the cytochrome *P*-450 species induced by phenobarbitone as a protein of mol.wt. 45 000 totally resistant to trypsin. However, since these workers purified cytochrome *P*-450 as the catalytically inactive form, cytochrome *P*-420, the possibility cannot be eliminated that their 'purified' preparation consisted largely of material partially degraded by proteolysis. This, coupled with the different electrophoretic system of analysis used, could explain the discrepancy observed in the molecular weights. Recent reports have demonstrated that a portion of newly biosynthesized cytochrome *P*-450 is translocated from the bound ribosomes across the membrane to the luminal contents (Craft *et al.*, 1978, 1979*a*,*b*) in contrast with the majority, which remains closely associated with the membrane. It is possible that these translocated species are destined for ultimate insertion into the membrane at a luminal-facing site.

The flavoprotein NADPH–cytochrome *P*-450 (cytochrome *c*) reductase, is necessary for the catalytic activity of cytochrome *P*-450. We have investigated the presence of this enzyme in membranes subjected to trypsin treatment. The low amounts of reductase activity observed after treatment of the membrane with trypsin are greatly enhanced by sodium deoxycholate at concentrations high enough to make the vesicles permeable to macromolecules, such as cytochrome *c*, and NADPH which otherwise cannot traverse the phospholipid bilayer. Furthermore, the activity present in untreated membrane can be also enhanced. This suggests that the NADPH–cytochrome *P*-450 (cytochrome *c*) reductase is also asymmetrically distributed across the transverse plane of the membrane and that a locus exists on both the cytosolic and luminal faces.

It is of interest to note that NADPH–cytochrome *P*-450 reductase has been reported to exist in two forms of different molecular weight (Coon *et al.*, 1977). Our finding that, under the conditions used, only 44% of the total cytochrome *P*-450 (detectable by dithionite reduction) can be directly reduced by NADPH appears to be at variance with the communication of Peterson *et al.* (1976) in which they report the almost total reduction of the cytochrome by NADPH. However, full details of the conditions that they employed are not given and it is not possible to resolve this discrepancy between their observations and our results. In contrast, other data (Mason *et al.*, 1965) has been presented, which, consistent with our observations, show a similar partial reduction of cytochrome *P*-450 by NADPH as compared with the cytochrome reducible by dithionite. We have demonstrated that the oxidized cytochrome *P*-450 remaining after treatment with NADPH can be reduced in the presence of a superoxide-generating system.

Since we demonstrate that cytochrome *P*-450 is asymmetrically distributed in the transverse plane of the membrane, it would seem reasonable to suggest that the cytochrome *P*-450 reducible by dithionite but not by NADPH is either located on the luminal face of the reticulum or is embedded in the phospholipid bilayer. It is this population of cytochrome *P*-450 that requires the presence of superoxide for reduction in the presence of NADPH. This superoxide-dependent reduction of cytochrome *P*-450 requires the presence of active NADPH–cytochrome *P*-450 (cytochrome *c*) reductase

Under the conditions employed, we have not been able to detect direct reduction of cytochrome *P*-450 by the superoxide ion. This finding differs from a previous report (Strobel & Coon, 1971), in which, by using a reconstituted system, the requirement for NADPH in the hydroxylation of benzphetamine and ethylmorphine could be replaced by superoxide. In the native membranes that we have used, no direct reduction can be detected. The apparent discrepancy could be due to a variety of factors involved in the reconstituted system. It is interesting to note that Strobel & Coon (1971) used a proteinase-solubilized preparation of the reductase in their reconstituted system and others have recently reported that such preparations cannot participate in the metabolism of substrates by a reconstituted cytochrome *P*-450-dependent system (Masters *et al.*, 1975).

The mechanism of action of superoxide in the NADPH-mediated reduction of cytochrome *P*-450 cannot at present be defined in molecular terms. Some reduction of cytochrome *P*-450 can also be achieved by superoxide in the presence of $NADP^+$ (results not shown). Thus superoxide reduction requires the presence of either the oxidized or the reduced form of NADP. There is the further possibility that superoxide may cause damage to the membrane vesicles, rendering them permeable to reagents. Further work is needed to elucidate this system.

We thank Carol Davie for the electron microscopy. We also thank the Cancer Research Campaign and the Medical Research Council for financial support and the Nuffield Foundation for provision of laboratory facilities.

References

Aust, S. D., Roerig, D. L. & Pederson, T. C. (1972) *Biochem. Biophys. Res. Commun.* **47**, 1133–1137

Bergman, A. & Dallner, G. (1976) *Life Sci.* **18**, 1083–1090

Blyth, C. A., Freedman, R. B. & Rabin, B. R. (1971) *Eur. J. Biochem.* **20**, 580–586

Chen. P. S., Toribara, T. Y. & Warner, H. (1956) *Anal. Chem.* **28**, 1786–1791

Coon, M. J., Haugen, D. A., Guengerich, F. P., Vermilion, J. L. & Dean, W. L. (1976) in *Structural Basis of Membrane Function* (Hatefi, Y. & Djavadi-Ohaniance, L., eds.), pp. 409–427, Academic Press, New York

Coon, M. J., Vermilion, J. L., Vatsis, K. P., French, J. S., Dean, W. L. & Haugen, D. A. (1977) in *Drug Metabolism Concepts* (Jerina, D. M., ed.), pp. 46–71, American Chemical Society, Washington

Craft, J. A., Cooper, M. B. & Rabin, B. R. (1978) *FEBS Lett.* **88**, 62–66

Craft, J. A., Cooper, M. B., Estall, M. R. & Rabin, B. R. (1979*a*) *FEBS Lett.* **98**, 403–407

Craft, J. A., Cooper, M. B., Estall, M. R., Rees, D. E. & Rabin, B. R. (1979*b*) *Eur. J. Biochem.* **96**, 379–391

Dallner, G., Siekevitz, P. & Palade, G. E. (1966) *J. Cell Biol.* **30**, 97–117

Ernster, L. & Orrenius, S. (1965) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **24**, 1190–1199

Folch, J., Lee, M. & Sloane-Stanley, G. H. (1957) *J. Biol. Chem.* **226**, 497–509

Franklin, M. R. & Estabrook, R. W. (1971) *Arch. Biochem. Biophys.* **143**, 318–329

Fridovich, I. (1962) *J. Biol. Chem.* **237**, 584–587

Fridovich, I. & Handler, P. (1958) *J. Biol. Chem.* **233**, 1581–1585

Gillette, J. R., Davis, D. C. & Sasme, H. A. (1972) *Annu. Rev. Pharmacol.* **12**, 57–84

Haugen, D. A. & Coon, M. J. (1976) *J. Biol. Chem.* **251**, 1817–1827

Haugen, D. A., Van der Hoeven, T. A. & Coon, M. J. (1975) *J. Biol. Chem.* **250**, 3567–3570

Ito, A. (1973) *J. Biochem. (Tokyo)* **75**, 787–793

Ito, A. & Sato, R. (1969) *J. Cell Biol.* **40**, 179–189

Kreibich, G., Debey, P. & Sabatini, D. D. (1973) *J. Cell Biol.* **58**, 436–462

Kuntzman, R. (1969) *Annu. Rev. Pharmacol.* **9**, 21–36

Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685

Leskes, A., Siekevitz, P. & Palade, G. E. (1971) *J. Cell Biol.* **49**, 264–287

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275

Lu, A. Y. H., Junk, K. W. & Coon, M. J. (1969) *J. Biol. Chem.* **244**, 3714–3721

Mason, H. S., Yamano, T., North, J. C., Hashimoto, Y. & Sakagishi, P. (1965) in *Oxidases and Related Redox Systems* (King, T. E., Mason, H. S. & Morrison, M., eds.), vol. 2, pp. 879–903, John Wiley and Sons, New York

Masters, B. S. S., Baron, J., Taylor, W. E., Isaacson, E. L. & Lo Spalluto, J. (1971) *J. Biol. Chem.* **246**, 4143–4150

Masters, B. S. S., Prough, R. A. & Kamin, H. (1975) *Biochemistry* **14**, 607–613

Mazur, A., Green, S. & Shorr, E. (1956) *J. Biol. Chem.* **220**, 227–235

Nilsson, O. S. & Dallner, G. (1977) *J. Cell Biol.* **72**, 568–583

Nilsson, O. S., DePierre, J. & Dallner, G. (1978) *Biochim. Biophys. Acta* **511**, 93–104

Omura, T. & Sato, R. (1964) *J. Biol. Chem.* **239**, 2379–2385

Orrenius, S., Berg, A. & Ernster, L. (1969) *Eur. J. Biochem.* **11**, 193–200

Peterson, J. A., Ebel, R. E. & O'Keefe, D. H. (1976) *Hoppe-Seyler's Z. Physiol. Chem.* **357**, 1049–1050

Phillips, A. H. & Langdon, R. G. (1962) *J. Biol. Chem.* **237**, 2652–2660

Rees, D. (1979) *Gen. Pharmacol.* **10**, 341–351

Strobel, H. W. & Coon, M. J. (1971) *J. Biol. Chem.* **246**, 7826–7829

Strobel, H. W., Lu, A. Y. H., Heidema, J. & Coon, M. J. (1970) *J. Biol. Chem.* **245**, 4851–4854

Takesue, S. & Omura, T. (1968) *Biochem. Biophys. Res. Commun.* **30**, 723–729

Vermilion, J. L. & Coon, M. J. (1974) *Biochem. Biophys. Res. Commun.* **60**, 1315–1322

Welton, A. F. & Aust, S. D. (1974) *Biochem. Biophys. Res. Commun.* **56**, 898–906

Welton, A. F., O'Neal, F. O., Chaney, L. C. & Aust, S. D. (1975) *J. Biol. Chem.* **250**, 5631–5639