†*Department of Toxicology*
Karolinska Institutet
Box 60 400
S-104 01 Stockholm
Sweden, and
‡*Department of Pathology*
Medical College of Ohio
C.S. 10008
Toledo, Ohio 43699
U.S.A.

INGRID NORDGREN*†
KATHLEEN BALDWIN‡
ROBERT FORNEY, JR.‡

* To whom correspondence should be sent.

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Cytochrome P-450-dependent lipid peroxidation in reconstituted membrane vesicles

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The flavoprotein NADPH-cytochrome P-450 reductase has mainly been regarded as responsible for producing reactive oxygen species such as the hydroxyl radical [1] or the superoxide anion radical [2], which have been proposed to act as initiators of liver microsomal lipid peroxidation: effective rates of peroxidation have been reached in systems where the reductase serves as electron donor to ADP-Fe^{3+} complexes. The hydroxyl radical [3], the perferryl ion [4, 5] and more recently a ferrous-dioxygen-ferric chelate [6] were proposed as initiating oxygen species. No role for cytochrome P-450 in the initiation step has hitherto been presented. However Svingen *et al.* [4, 7] have suggested a role for cytochrome P-450 in the lipid hydroperoxide-dependent initiation, whereby the peroxidative capacity of cytochrome P-450 was suggested to promote this type of initiation by producing peroxide-derived products such as peroxy or alkoxyradicals, hydroxyl radicals [7, 8] or singlet oxygen [9] that may contribute to chain propagation.

It was recently found that cytochrome P-450 itself produces reactive oxygen radicals [10–14]. These findings suggested that there might exist a cytochrome P-450-dependent lipid peroxidation mechanism utilizing active oxygen species as initiators. Since most studies concerning reconstituted systems and lipid peroxidation have been performed in the presence of high concentrations (>100 μM) of chelated iron, it was considered of importance to evaluate any role for cytochrome P-450 in lipid peroxidation using reconstituted membrane systems fortified with NADPH in a system free from exogenous iron and iron chelators.

Materials and methods. Desferoxamine was from CIBA. Diethylenetriaminepentaacetic acid (DETAPAC) was purchased from Sigma, whereas EDTA was from Fluka. Chelex 100 was obtained from Bio-Rad. Microsomal phospholipids were extracted from liver microsomes of phenobarbital-treated rabbits according to Bligh and Dyer [15] and stored under nitrogen in sealed tubes at −20°. Electrophoretically homogeneous preparations of NADPH-cytochrome P-450 reductase and cytochromes P-450 LM2 and P-450 LM4 from liver microsomes of phenobarbital-treated rabbits were prepared according to methods of Yasukochi and Masters [16] and Haugen and Coon [17], respectively. The ethanol and benzene-inducible form of rabbit liver microsomal cytochrome P-450 (P-450 LMeb) was purified essentially as previously described [18]. The specific contents of the enzyme preparations used were: P-450 LM2, 10.5–13 nmole/mg; P-450 LMeb, 11–14.3 nmole/mg; P-450 LM4, 14.0 nmole/mg; NADPH-cytochrome P-450 reductase, 20–22 nmole of flavin per mg of protein.

Unilamellar phospholipid vesicles containing microsomal phospholipids, cytochrome P-450 and NADPH-cytochrome P-450 reductase in a molar ratio of 1200:1:0.4 were prepared by the cholate gel filtration technique [19] in Chelex 100-treated 15 mM potassium phosphate buffer, pH 7.4, containing 50 mM KCl.

Reaction mixtures contained vesicles corresponding to 0.1 nmole of cytochrome P-450 in chelexed 15 mM potassium phosphate buffer, pH 7.4, containing 50 mM KCl in a final volume of 1 ml. Preincubation was performed for 15 min at 30° and the incubations were started by the addition of 10 μl 10 mM NADPH and terminated by the addition of 0.25 ml 40% trichloroacetic acid and 0.125 ml of 5 M HCl. The incubation time was generally 30 min. Lipid peroxidation products were measured using the thiobarbituric acid assay [20]. The extinction coefficient used was 156 mM^{-1} cm^{-1}. Control incubations were carried out by adding NADPH after the addition of trichloroacetic acid.

Results and discussion. Incubation of membrane vesicles containing cytochrome P-450 LM2 and NADPH-cytochrome P-450 reductase in a chelexed, EDTA-free reaction system fortified with NADPH, resulted in a time-dependent production of TBA-reactive substances (Fig. 1a). The process was linear for at least 15 min. Linearity was also reached in response to increasing amounts of vesicles (Fig. 1b). About half the rate of lipid peroxidation was registered using membranes containing the ethanol and benzene-inducible form of rabbit liver microsomal cytochrome P-450 (P-450 LMeb), whereas the cytochrome P-450 LM4 fraction was almost equally effective as P-450 LM2 in catalyzing the generation of TBA-reactive material in the reconstituted membranes (Table 1). When vesicles devoid of cytochrome P-450 were used, only 5% of the rate of lipid peroxidation was detected (Table 1). In order to evaluate the kind of cytochrome P-450 participation in the reaction, vesicles devoid of P-450, but only containing P-450 reductase, were supplemented with boiled preparations of P-450 LM2. In this case, an inhibition of the rate of formation of TBA-reactive material, compared to P-450-containing vesicles, was registered (not shown in the figure).

Membrane vesicles were prepared having variable amounts of cytochrome P-450 LM2, whereas the content of NADPH-cytochrome P-450 reductase was kept constant.

The rate of lipid peroxidation, as measured by the TBA method, increased until a 1:1 molar ratio between the protein components was reached in the membrane (Fig. 2) and was not further altered in response to added cytochrome P-450. The iron chelators DETAPAC and EDTA effectively inhibited both the cytochrome P-450 LM2-dependent and NADPH-cytochrome P-450 reductase-dependent lipid peroxidation in the reconstituted membrane vesicles (Table 2). Desferoxamine almost completely scavenged the P-450-reductase-dependent peroxidation but only slightly lipid peroxidation detected in membranes also containing cytochrome P-450 LM2. Addition of $FeCl_3$ up to 20 μM concentration did not increase the rate of lipid peroxidation detected in the absence of chelators by more than 20% (not shown).

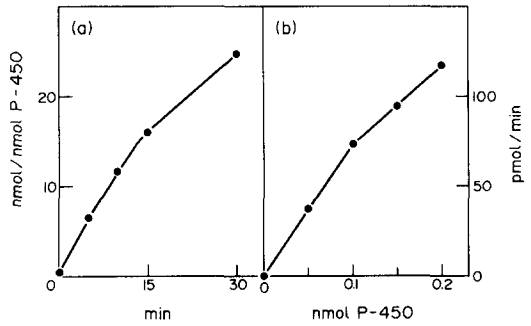

Fig. 1. Lipid peroxidation, as revealed by the formation of TBA-reactive material, in reconstituted cytochrome P-450 LM2-containing phospholipid vesicles as a function of time (a) and the amount of membranes in the incubation system (b). Incubations were carried out as described under Experimental with vesicles corresponding to 0.1 μM cytochrome P-450 LM2, 0.04 μM NADPH-cytochrome P-450 reductase and 120 μM microsomal phospholipids for 30 min at 30° unless otherwise stated.

Table 1. Cytochrome P-450 and NADPH-cytochrome P-450 reductase-dependent lipid peroxidation in reconstituted membrane vesicles as revealed by the formation of TBA-reactive products

Enzyme	TBA-reactive products (pmol/nmol P-450, min)
P-450 LM2	733 ± 134 (18)
P-450 LMeb	390 (3)
P-450 LM4	560 (2)
P-450 reductase	36 ± 19 (4)*

Incubations were performed as outlined under Experimental with vesicles corresponding to a final concentration of 120 μM phospholipids, 0.1 μM cytochrome P-450 and/or 0.04 μM P-450 reductase

The number of experiments are given in parentheses.

* pmol/0.5 nmol reductase, min.

The results presented indicate the existence of a cytochrome P-450-dependent lipid peroxidation mechanism. The rate of generation of TBA-reactive products in reconstituted membrane vesicles was 10–20-fold higher in systems containing cytochrome P-450 than in membranes devoid of P-450. The saturation of the rate of lipid peroxidation reached at 1:1 molar ratio between P-450 reductase and P-450 indicates that the action of P-450 is mediated via electron transport and not by the peroxidative capacities of the enzyme. This is further supported by the fact that P-450 LM4 was as active as the other cytochromes P-450, despite being a very poor peroxidase [21].

Special emphasis was made to work in systems depleted from interfering components. Thus all buffers and enzyme

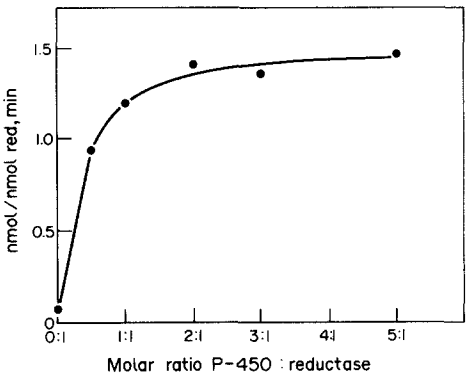

Fig. 2. Lipid peroxidation, as revealed by the formation of TBA-reactive material in reconstituted phospholipid vesicles as a function of the amount of cytochrome P-450 LM2 in the membranes. Incubations were performed as described under Experimental. The reaction mixtures contained membrane vesicles corresponding to 0.05 μM NADPH-cytochrome P-450 reductase and indicated amounts of cytochrome P-450 LM2. The results are the mean values from 4 different experiments performed with 2 different enzyme preparations.

Table 2. Effect of iron chelators on the rate of formation of TBA-reactive products in reconsistuted membrane vesicles containing cytochrome P-450 LM2 and/or NADPH-cytochrome P-450 reductase

Chelator used		TBA-reactive products (pmol/nmol, min) LM2	% Inh.	Reductase	% Inh.
None		733	—	75	—
Desferoxamine	10 μM	751	—		
	100 μM	436	40	8	89
EDTA	1 μM	668	9		
	5 μM	63	91		
	10 μM	25	97		
	100 μM	31	96	15	79
DETAPAC	10 μM	46	94		
	100 μM	31	96	13	83

Incubations were performed with vesicles corresponding to 0.1 nmol P-450 and/or 0.05 nmol P-450 reductase as outlined under Experimental.

The data represent mean values of 2–4 different experiments.

solutions were cleaned using Chelex-100. Previous results have shown that contaminating iron does not exceed a concentration of about 1 μM in such a system [22]. Nevertheless, the residual iron present seems to be sufficient to catalyze the lipid peroxidation observed: addition of the iron chelators EDTA and DETAPAC effectively inhibited the process. One may assume that cytochrome P-450 either directly, or indirectly via generation of superoxide anions, reduces non-heme iron that may act as initiator of the lipid peroxidation after binding of oxygen.

In conclusion, the results presented indicate that cytochrome P-450 may contribute to initiation of lipid peroxidation, although the extent of contribution of P-450 to the lipid peroxidation by its peroxidative properties requires further work.

Present studies in our laboratory focus on the mechanism of the cytochrome P-450-dependent lipid peroxidation.

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Department of Physiological Chemistry
Karolinska Institute
P.O. Box 60 400
S-104 01 Stockholm, Sweden

GUNILLA EKSTRÖM
MAGNUS INGELMAN-SUNDBERG

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The influence of some anthelmintics on the bioenergetic metabolism of *Trichinella spiralis* and *Trichinella pseudospiralis*

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The goal of this investigation was to identify the drug sensitivities of two related nematodes: *Trichinella spiralis* and *Trichinella pseudospiralis*. The focus was upon the bioenergetic metabolism of *T. spiralis* and *T. pseudospiralis* [1]. Some of the results presented in this paper have been published as abstracts [2, 3].

Of the drugs effective in the therapy of intestinal nematodes and tapeworms, the most frequently investigated have been levamisole*, mebendazole† and praziquantel‡. Early studies demonstrated the inhibition of succinate dehydrogenase-fumarate reductase (SDH-FR) complex by thiabendazole§, levamizole and praziquantel. Today, the mode of action of these drugs is considered to be more complex [4]. For example, levamisole affects the neuromuscular system of Ascaris [5] and, in high doses, inhibits fumarate reductase [6]. Immature and adult nematodes kept in tetramisole and levamisole solutions show spastic contraction followed by tonic paralysis. The effect could be either reversible or irreversible according to the worm species. It has been suggested [6] that, in some nematodes, the irreversibility of this neuromuscular blocking activity may be related to the inhibition of the fumarate reductase.

Köhler and Bachman [7] localized the site of the inhibitory action in Ascaris of levamisole, thiabendazole, praziquantel and chloroquine in the electron transport chain between a quinone and NADH-dehydrogenase.

These drugs, except thiabendazole, did not affect succinate oxidation and reduction. Recently, Prichard *et al.* [8] concluded that the sustained release of endogenous Ca^{2-} by the drugs may affect the sequence of excitation-contraction coupling, and cause observed contraction of *H. dimunta*. The biochemical and neuropharmacological changes due to the praziquantel (in conc. of 10^{-7}M) were

* Levamisole is the laboratory isomer of 2,3,5,6-tetrahydro-6-phenyl imidazo(2,1-b) thiazole (the racemic mixture is known as tetramisole).

† Mebendazole is methyl-5(6)-benzoly-2-benzimidazole carbamate.

‡ Praziquantel is 2-cyclohexylcarbonyl-1,3,4,6,7,11b-hexahydro-2H-pyrazino(2,1-a)isoquinoline-4-one.

§ Thiabendazole is 2-(thiazol-4′-yl)benzimidazole.