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# STUDIES ON THE LOCALIZATION OF REACTION SITES WITHIN THE REDUCED NICOTINAMIDE-ADENINE DINUCLEOTIDE PHOSPHATE-OXYGENASE SYSTEM OF RAT LIVER MICROSOMES FOR THE *N*- AND C-OXIDATION OF DIMETHYLANILINE AND FOR THE PEROXIDATION OF UNSATURATED FATTY ACIDS

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**Abstract**—Studies on the localization within the NADPH-oxygenase system of rat liver microsomes of the reaction site for the *N*-oxidation of dimethylaniline indicate that only the initial component of the electron transport chain, can participate in the reaction. The results support the hypothesis that cytochrome P-450 is essential for the NADPH-dependent peroxidation of unsaturated fatty acids by endoplasmic reticulum, as well as for the C-oxidation of dimethylaniline. Ferrous pyrophosphate, when used as a cofactor to couple NADPH oxidation to the peroxidation of unsaturated fatty acids, produces spectral changes very similar to the absorption spectrum of the cytochrome P-450-carbon monoxide complex.

The results further indicate that there are two modes of formaldehyde formation from dimethylaniline: the first, by means of direct oxidation of the C-atom of the methyl group, without participation of dimethylaniline-*N*-oxide, and the second from dimethylaniline-*N*-oxide by means of transfer of oxygen from the *N*- to the C-atom.

THE OXIDATION of NADPH by the enzyme systems of the endoplasmic reticulum is accompanied by activation of molecular oxygen. The activated oxygen thus generated is utilized in the *N*- and C-oxidation of xenobiotics and of some natural substrates.[1–3] In the presence of ferrous pyrophosphate (FePP), the oxidation of NADPH results in peroxidation of unsaturated fatty acids (UFA) of microsomal lipids.[4–6]

It is now established that the terminal oxidase of the NADPH-dependent cytochrome P-450 enzyme system serves as a site for C-atom oxidation;[7,8] however, the sites within the microsomal electron transport chain for *N*-atom oxidation and for UFA peroxidation have not yet been elucidated.[5,9,10]

In the present study the possibility of assignment to specific components of the electron transport chain (ETC) of the reactions of *N*- and C-oxidation of dimethylaniline (DMA) and the peroxidation of UFA was investigated by means of fractionation of microsomes, the induction of microsomal drug-metabolizing systems and the use of agents which alter the rate of electron transfer within different parts of the chain.

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## MATERIALS AND METHODS

Male rats weighing 200–250 g were used. The microsomal fraction was obtained as previously described,[11] except that the last centrifugation was performed on the VAC-60 centrifuge at 105,000 *g* for 1 hr. The electron transfer membranes (ETM) of rat liver microsomes were isolated as described by Maclennan *et al.*[12] For induction of microsomal drug-metabolizing activity, the animals were given three daily intraperitoneal injections of phenobarbital (PB), 100 mg/kg. The rats were killed 24 hr after the last injection.

The specific activities of the enzyme systems responsible for *N*- and C-oxidation of DMA were determined by method of Pettit and Ziegler,[13,14] measuring the rate of the first process by the rate of formation of formaldehyde (FA) and that of the second by the rate of formation of the *N*-oxide of DMA (NO). The activity of the NADPH- and ascorbate-dependent systems of UFA peroxidation (NDS and ADS) was determined by the method of Hochstein and Ernster[4] and of Archakov *et al.*[11] The activity of NADPH-neotetrazolium reductase and the rate of NADPH-dependent oxygen uptake were measured by methods described earlier.[11] NADPH-oxidase was assayed as described by Estabrook *et al.*[15] All specific activities are expressed as nanomoles of product formed per minute per milligram of microsomal protein.

Cytochrome $b_5$ and P-450 levels were determined utilizing the SF-10 spectrophotometer as described earlier.[16] Substrate-induced changes in the spectrum of cytochrome P-450 were determined by the method of Estabrook *et al.*[17] The microsomal fraction for the latter studies was obtained from rats which had been given PB (80 mg/kg) intraperitoneally for 3 days. The animals were killed 48 hr after the third administration.

Protein content was measured by the method of Lowry *et al.*[18]

*Chemicals.* Sources of reagents were as follows: NADPH and NADP: CalBiochem; SKF 525-A, [2-(diethylamine) ethyl 2,2-diphenylpropilacetate hydrochloride]: Smith, Kline & French Laboratories; 3-methylcholanthrene: Fluka a.g., Switzerland; Triton X-100: Schuchardt, Munich; menadione: Berlin-Chemi. All other chemicals were obtained from local sources.

## RESULTS AND DISCUSSION

*Subfractionation of microsomes.* Treatment of microsomes with isoamyl alcohol and consequent centrifugation of this preparation yielded a membrane fraction containing the enzymes of electron transfer at a specific activity two to three times higher than that of the initial preparation, except for the system of *N*-oxidation of DMA (Table 1). Thus, *N*-oxidation of DMA is the only type of enzyme activity which is lower in electron transfer membranes (ETM) than in the original microsomes. It is possible therefore that one component of the latter system is not present in the ETM.

Taking into consideration these results, it was of interest to determine the correlation between *N*- and C-oxidation of DMA during PB induction of microsomal drug-metabolizing enzymes (Table 2). It is evident from Table 2 that induction by PB produces an increase only in the rate of C-oxidation of DMA. The rate of *N*-oxidation remains unchanged. These findings lead us to the preliminary conclusion that *N*- and C-oxidation processes are independent from each other and are located in different parts of the NADPH-oxygenase chain.

TABLE 1. ENZYME ACTIVITIES AND CYTOCHROME CONTENTS OF MICROSOMAL ELECTRON TRANSFER MEMBRANES*

| Type of activity | Microsomes | ETM | Pellet |
|---|---|---|---|
| C-oxidation of DMA | 11·9 | 27·8 | 3·10 |
| *N*-oxidation of DMA | 3·80 | 1·79 | 0·32 |
| NADPH-dependent peroxidation of UFA | 1·38 | 5·60 | 0·20 |
| Ascorbate-dependent peroxidation of UFA | 2·80 | 9·47 | 0·50 |
| NADPH-neotetrazolium reductase | 77·4 | 245·0 | 41·8 |
| NADPH-oxidase | 2·00 | 7·70 | 1·00 |
| Cytochrome P-450 content $\frac{\Delta D_{450-490}}{\text{mg protein}} \cdot 10^2$ | 3·15 | 5·63 | 0·53 |
| Cytochrome $b_5$ content $\frac{\Delta D_{408-428}}{\text{mg protein}} \cdot 10^2$ | 3·93 | 15·0 | 2·73 |
| Protein content (mg/ml) | 17·3 | 3·24 | 7·60 |

* Average data from four experiments (nmoles.mg$^{-1}$.min$^{-1}$).

TABLE 2. RATES OF C- AND *N*-OXIDATION OF DMA, AND CYTOCHROME CONTENTS OF LIVER MICROSOMES OF RATS PRETREATED WITH PHENOBARBITAL*

| Type of activity | Controls | Phenobarbital pretreated | P value |
|---|---|---|---|
| C-oxidation of DMA (nmoles.mg$^{-1}$.min$^{-1}$) | 19·7 | 40·5 | 0·001 |
| *N*-oxidation of DMA (nmoles.mg$^{-1}$.min$^{-1}$) | 4·03 | 3·58 | 0·001 |
| Cytochrome P-450 $\frac{\Delta D_{450-490}}{\text{mg protein}} \cdot 10^2$ | 2·96 | 5·06 | 0·001 |
| Cytochrome $b_5$ $\frac{\Delta D_{408-428}}{\text{mg protein}} \cdot 10^2$ | 3·37 | 3·04 | 0·01 |

* Average data from four experiments.

In order to determine which components of ETC are responsible for the processes under study, we have used a method of inhibitor analysis. For this purpose, the ETC was divided into three components: the initial: NADPH-flavoprotein-reductase; the middle: flavoprotein-reductase-X (cytochrome $b_5$?) and the terminal: cytochrome P-450.

*Initial component of ETC.* The results presented in Table 3 show clearly that the initial part of the ETC plays different roles in the processes under study. The most characteristic feature is the identical effect of the inhibitors studied on the rate of C-oxidation of DMA and the peroxidation of UFA in contrast to the different effects of these agents on the process of *N*-oxidation. The absence of formaldehyde and malonic aldehyde (MA) formation under conditions in which a considerable accumulation of *N*-oxide of DMA is seen while using NADH as a substrate serves as an illustration of this differential effect.

TABLE 3. INFLUENCE OF CHANGES IN THE RATE OF ELECTRON TRANSFER IN THE INITIAL, MIDDLE AND TERMINAL PARTS OF THE NADPH-OXYGENASE CHAIN ON RATES OF *N*- AND C-OXIDATION OF DMA AND PEROXIDATION OF UFA (% OF CONTROL)

| Components of electron transfer chain | Additions to standard mixture | Type of oxidation | | | |
|---|---|---|---|---|---|
| | | *N*-oxidation | C-oxidation | NADPH-dependent peroxidation of UFA | Ascorbate-dependent peroxidation of UFA |
| NADPH-FP-reductase | Control (standard mixture) | 100 | 100 | 100 | 100 |
| | NADH,* 3 mM | 73 | 27 | 17 | |
| | NADP, 3 mM | 58 | 70 | 72 | |
| | Menadione | | | | |
| | 0·2 mM | 45 | 20 | 5 | 78 |
| | 0·7 mM | | 10 | 0 | 67 |
| | Catalase | | | | |
| | 160 mg | 144 | 58 | 72 | |
| | 480 mg | 110 | 36 | 33 | 20 |
| | Amytal | | | | |
| | 2 mM | 130 | 100 | 100 | 100 |
| FP-reductase-X (cyt. $b_5$?) | PCMB, 0·16 mM | 76 | 16 | 30 | 100 |
| Cytochrome P-450 | CO | 85 | 0 | 100 | 100 |
| | NaCN, 1 mM | 100 | 40 | 44 | 84 |
| | 10 mM | 100 | 23 | 0 | 0 |
| | Propazin | | | | |
| | 0·5 mM | 90 | 60 | 0 | 0 |
| | DMA, 5 mM | | | 0 | 0 |
| | DMA, 5 mM + CO | | | 0 | 0 |
| | AP, 5 mM | | | 0 | 0 |
| | AP, 5 mM + CO | | | 0 | 0 |
| | Nicotinamide | | | | |
| | 200 mM | | 30 | 60 | 100 |
| | SKF 525-A | | | | |
| | 0·1 mM | | | 10 | |

* NADH was used instead of NADPH.

Catalase, by removing hydrogen peroxide, shifts the reaction toward additional peroxide formation from the oxidation of flavoprotein (FP). Thus, demethylation is inhibited and the rate of *N*-oxidation is increased at the same time.

Amytal, while having no effect on the rate of formation of MA and formaldehyde, increases the rate of formation of the *N*-oxide of DMA. Menadione, receiving electrons from FP, inhibits strikingly C-oxidation of DMA and NADPH-dependent peroxidation, but does not have an effect on *N*-oxidation. These results indicate that the processes of C-oxidation of DMA and NDS of UFA utilize the same components of the NADPH-FP-reductase enzyme system.

More complex is the question of the formation of the *N*-oxide of DMA. Taking into consideration the acceleration of this process under the influence of catalase and amytal, together with the effectiveness of NADH while using DMA as substrate, it would appear that the oxidation of the nitrogen of DMA is closely connected with the formation of hydrogen peroxide at the flavoprotein level. Failure of previous

attempts to model the formation of the *N*-oxide of DMA in artificial systems containing or forming $H_2O_2$ indicates that this process is not a result of a simple oxidation of DMA by $H_2O_2$, but requires the participation of an unknown factor, possibly a peroxidase. This conclusion could explain the results of our experiments with ETM and induction.

*Middle component of ETC.* The results of the study of the influence of p-chloromercuribenzoate (PCMB) on *N*- and C-oxidation of DMA and peroxidation of UFA (Table 3) confirm the earlier statement regarding the localization of the formation of the *N*-oxide of DMA to the initial component of the chain of NADPH oxidation. This compound, inhibiting electron transport at the segment behind FP, slows only insignificantly the speed of *N*-oxidation while inhibiting sharply both C-oxidation and NDA of UFA.

*Terminal component of ETC.* It can be seen from Table 3 that the compounds, interacting with cytochrome P-450, affect differently the activity of the systems under study. The following characteristic features of their action should be stressed: (1) None of these compounds inhibits to a significant extent the rate of *N*-oxidation. (2) Carbon monoxide, which binds to the heme of cytochrome P-450, does not affect the rate of peroxidation. (3) All compounds that interact with the apoenzyme of cytochrome P-450 inhibit peroxidation. (4) C-oxidation is blocked by all those inhibitors which interact with both the heme and the apoenzymic part of cytochrome P-450.

The effect of substrates for demethylation reactions on activity of peroxidation systems should also be noted. Inhibition of NDS by aminopyrine was observed by Hochstein and Ernster[4] and was interpreted by them as competition for NADPH. It was subsequently found, however, that AP and DMA also have an inhibiting effect on ascorbate-dependent peroxidation, for which NADPH is not required. These data permit an alternative explanation for the inhibition of peroxidation by substrates for demethylation. If this effect depends not on the competition for NADPH but on the binding of substrates to cytochrome P-450, then CO, supressing metabolism of DMA and aminopyrine should not present the inhibition of NDS by the latter compounds. The absence of influence of CO on the inhibiting effect of DMA and aminopyrine, nicotinamide, SKF 525-A and NaCN confirm this hypothesis.

*Interaction of inhibitors with cytochrome P*-450. In the course of spectrophotometric investigations of the binding to cytochrome P-450 of compounds which affect the rate of metabolism of DMA and of peroxidation of UFA, it was observed (Fig. 1) that most of the inhibitors which block peroxidation cause significant spectral changes as a result of their interaction with cytochrome P-450 (curves 5–9). It is characteristic, however, that all of them, while inhibiting peroxidation and C-oxidation, are without effect on the rate of formation of the *N*-oxide of DMA.

Menadione and EDTA do not interact with the oxidized form of cytochrome P-450. FePP does not bind the oxidized form of cytochrome P-450, but is capable of causing spectral changes in the presence of reduced NADP (Fig. 2).

From Fig. 2 it can be seen that the addition of FePP causes the formation of a characteristic absorption maximum at 450 nm, even in the absence of CO. Under these conditions, the kinetics of reduction of this pigment and of cytochrome $b_5$ change markedly.

When NADH is used as electron donor, the formation of this peak at 450 nm is not observed. The observed formation of a CO-cytochrome P-450-like complex

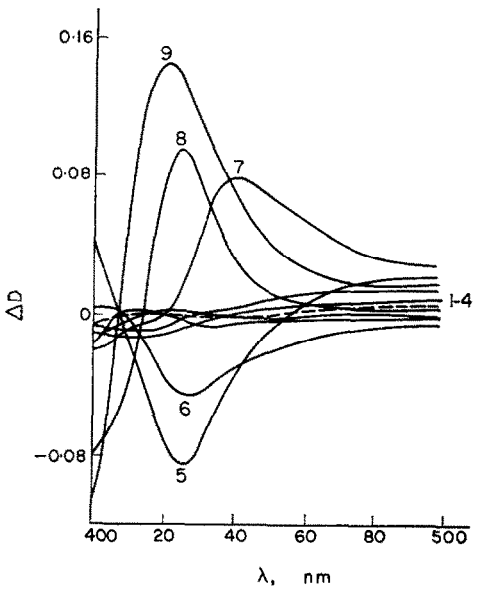



FIG. 1. Change of difference spectrum of microsomes in the presence of FePP and of inhibitors of peroxidation of FUA. (1) FePP (0·024 mM $Fe^{2+}$ and 0·4 mM NaPP); (2) EDTA, 3 mM; (3) propazine, 0·1 mM; (4) menadione, 0·3 mM; (5) DMA, 5 mM; (6) PCMB, 0·17 mM; (7) diethylparaphenylenediamine, 0·07 mg/ml; (8) nicotinamide, 200 mM; (9) NaCN, 1 mM.

can be explained either by direct interaction of ferrous pyrophosphate with reduced cytochrome P-450 or by formation of CO during the peroxidation of UFA. The second assumption seems more feasible in view of the results of Remmer *et al.*[7]

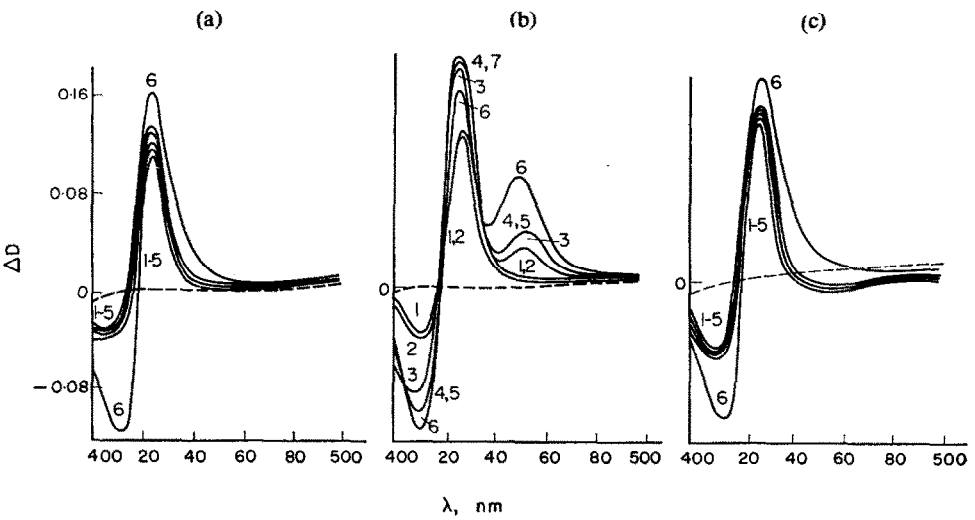



FIG. 2. Influence of FePP on the kinetics of reduction of cytochromes $b_5$ and P-450 (a) in the absence of FePP; (b) FePP was added in concentrations: 0·024 mM $Fe^{2+}$ and 0·4 mM NaPP with NADPH as substrate; (c) FePP was added in concentrations: 0·024 mM $Fe^{2+}$ and 0·4 mM NaPP with NADH as substrate. Time after addition of substrate: (1) 1 min; (2) 3 min; (3) 5 min; (4) 7 min; (5) 9 min; (6) dithionite added at 12 min after addition of substrate.

*Effect of detergents.* Taking into account the necessity of the whole ETC and the special role of cytochrome P-450 for the processes of C-oxidation of DMA and NADPH-dependent peroxidation of UFA, we can predict the effect of detergents on these systems.

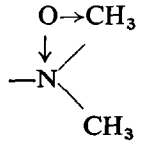
Triton X-100, by solubilizing the components of the electron transfer chain and converting cytochrome P-450 to its inactive form cytochrome P-420, will decrease the rate of formation of formaldehyde and MA, exerting, however, no inhibiting effect on the rate of *N*-oxidation of DMA, for which the flavoprotein component is needed. It is seen from Table 4 that the observed results are in good conformity with those predicted.

TABLE 4. INFLUENCE OF TRITON X-100 ON THE RATES OF *N*- AND C-OXIDATION OF DMA AND PEROXIDATION OF UFA (% OF CONTROL)

| | Type of oxidation | | | |
|---|---|---|---|---|
| | *N*-oxidation | C-oxidation | NADPH-dependent peroxidation of UFA | Ascorbate-dependent peroxidation of UFA |
| Control | 100 | 100 | 100 | 100 |
| Triton X-100 (0·3 mg/mg protein) | 100 | 45 | 62 | 56 |

Up to the present time, the view of Ziegler and Pettit[9] has been generally accepted, that the *N*-oxide of DMA is an intermediate in formaldehyde formation. However, an accumulation of the *N*-oxide of the DMA-intermediate product accompanied by a decrease in the rate of formaldehyde formation (final product), taken by Ziegler and Pettit as evidence for this hypothesis, was not observed in our study. The results of the present study are compatible with the hypothesis that the *N*-oxide of DMA is formed at the flavoprotein stage of the ETC and that formaldehyde is not a product of its transformation. These observations are in agreement with the results of Hlavica and Kiese.[19]

The inhibition of formaldehyde formation by CO and SKF 525-A when using the *N*-oxide of DMA instead of DMA as substrate (the reaction goes in the absence of NADPH and $O_2$) indicates that this process is catalyzed by cytochrome P-450.[10] It would follow therefore that the *N*-oxide of DMA, as well as DMA itself, should be bound by the apoenzymic part of cytochrome P-450. Thus, two routes of formaldehyde formation from the methyl group of DMA are possible: (1) by means of the primary oxidation of the C-atom of $CH_3$ without participation of the *N*-oxide of DMA; and (2) from the *N*-oxide of DMA as a result of transfer of oxygen from the *N*- to the C-atom



Both processes are catalyzed by cytochrome P-450.

The existence of similar effects of most inhibitors on the processes of C-oxidation of DMA and of NADPH-dependent peroxidation of UFA supports the hypothesis of direct participation in both these processes of all components of the chain of oxidation of NADPH, including cytochrome P-450.

It has been possible to provide additional support for this hypothesis by the use of substrates of demethylation reactions in the presence of CO, nicotinamide, NaCN and SKF 525-A as peroxidation inhibitors.

The absence of a CO effect on peroxidation would appear therefore to indicate different mechanisms for the participation of cytochrome P-450 in the processes of UFA- and of C-oxidation, but not as negating the role of cytochrome P-450 in these processes.

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