IODINE- AND CHLORINE-CONTAINING OXIDATION AGENTS AS HYDROXYLATING CATALYSTS IN CYTOCHROME *P*-450-DEPENDENT FATTY ACID HYDROXYLATION REACTIONS IN RAT LIVER MICROSOMES

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1. Introduction

Following the discovery that cytochrome *P*-450 of liver microsomes can catalyze organic hydroperoxide-supported hydroxylation of various substrates in the absence of NADPH and molecular oxygen [1–5] work in our laboratory has shown that $NaClO_2$ and $NaIO_4$ support the hydroxylation of steroid substrates in liver microsomes [4,5], in partially purified cytochrome *P*-450 preparations obtained from liver microsomes [6] and from *Bacillus megaterium* [7] and in adrenocortical microsomal and mitochondrial preparations [8]. The findings that $NaClO_2$ and $NaIO_4$ may serve as oxygen donors in cytochrome *P*-450-catalyzed hydroxylation reactions indicate that the active oxygenated species of the cytochrome contains a single oxygen atom. In order to further examine mechanisms involved in cytochrome *P*-450-catalyzed oxygenation reactions we have in this study investigated oxidation agent-supported hydroxylations of fatty acids. As an extension of our previous work we have also tried to find more efficient hydroxylating agents with iodine as central atom and have found iodosobenzene and iodosobenzene diacetate to be highly efficient oxygen donors in ω2-hydroxylation of fatty acids.

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2. Materials and methods

[1-^{14}C]Laurate, [1-^{14}C]palmitate and [1-^{14}C]stearate were purchased from The Radiochemical Centre (Amersham, England) and had specific radioactivities of 29, 56 and 58 mCi/mmol, respectively. Sodium chlorite and sodium periodate were obtained from commercial sources. Iodosobenzene (φIO), iodosobenzene diacetate ($\varphi I(OAc)_2$) and iodoxybenze (φIO_2) were synthesized according to Saltzman and Sharefkin [9], Swaminathan and Venkatasubramanian [10] and Lucas and Kennedy [11], respectively. NADP was purchased from Sigma Chemical Co. (St. Louis, Mo., USA).

Microsomes were prepared from livers of male Sprague-Dawley rats (220–250 g). The animals were killed by cervical dislocation and the livers were taken out immediately and chilled on ice. Liver homogenates, 50% (w/v) were prepared in a modified Bucher medium [12], pH 7.4, with a Potter-Elvehjem homogenizer equipped with a loosely fitting Teflon pestle. The homogenate was centrifuged at 20 000 × *g* for 15 min. The microsomal fraction was obtained by centrifuging the 20 000 × *g* supernatant fluid at 105 000 × *g* for 70 min. The microsomal fraction was suspended in 12 ml of the homogenizing medium and was homogenized with a loosely fitting pestle. The resulting suspension was recentrifuged at 105 000 × *g* for 70 min and the obtained sediment was suspended and homogenized in 36 ml of Bucher medium and used for incubations. Standard incubation conditions were as follows: ^{14}C-labelled fatty acid substrates were

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diluted with unlabelled fatty acid in acetone prior to use. A 50 μl solution (400 μg, 1 X 10^6 cpm) was added to a 2 ml final volume containing microsomes (1 mg of protein/ml) and Bucher medium. Oxidation agent-supported reactions were started by adding the following final concentration of reagent: $NaClO_2$ (10 mM), $NaIO_4$ (10 mM), φIO (5 mM), $\varphi I(OAc)_2$ (5 mM) or φIO_2 (5 mM). NADPH-supported reactions were started by adding 1 mM (final concentration) NADP and an NADPH-regenerating system [13]. The incubations were performed in air at 37°C for 2 s up to 15 min and reactions were terminated with 1 ml of 96% (v/v) aqueous ethanol. The mixture was then diluted with 10 ml 0.01 M HCl and extracted three times with 10 ml of ethyl acetate. The ethyl acetate extracts were washed with distilled water until neutral, taken to dryness under nitrogen, methylated with diazomethane and analyzed by radio–gas chromatography using a Barber-Colman 5000 instrument equipped with a 1% OV-17 column. The metabolites were identified by gas chromatography – mass spectrometry carried out on an LKB 2091 instrument. Mass spectra were recorded on magnetic tape following on-line treatment in a PDP 11/10 computer [14]. Hydroxylated fatty acids were quantitated from the radio–gas chromatograms by peak area measurement (triangulation). Reaction rates were calculated from the linear portion of the curve and expressed as nmoles hydroxylated product formed/min/mg protein.

3. Results

Whereas NADPH supported both ω1- and ω2-hydroxylation of laurate, palmitate and stearate, as previously described by Björkhem and Danielsson [15], the oxidation agents under investigation only supported ω2-hydroxylation of these fatty acids (fig.1a–c). No hydroxylated fatty acids were formed during control incubations with fatty acid substrate, oxidation agent and boiled liver microsomes (fig.1c).

NADPH-supported hydroxylation reactions were linear with time at least up to 15 min (fig.2) whereas $NaIO_4$-, φIO- $\varphi I(OAc)_2$-supported ω2-hydroxylation was linear with time only during the first few seconds (fig.3). $NaClO_2$-supported ω2-hydroxylation was linear with time during 1–2 min (data not shown). Saturation with oxidation agent was obtained at 5–10 mM concentration (fig.4).

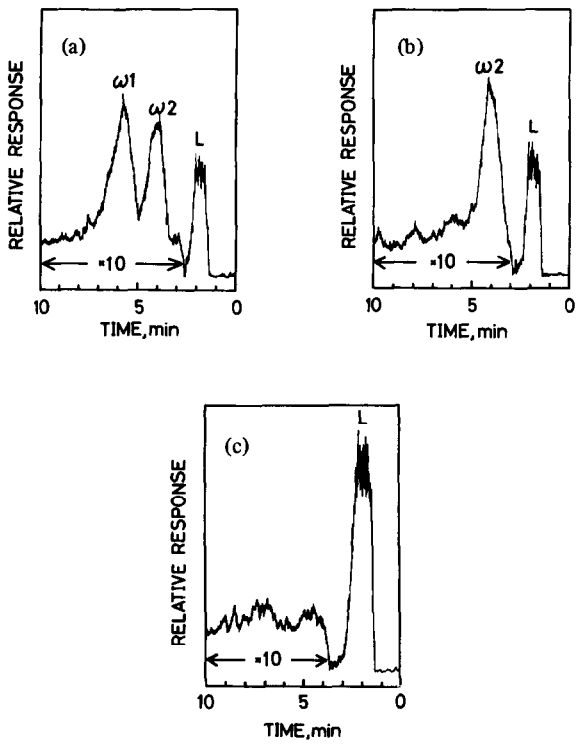

Fig.1. Radio–gas chromatographic analyses of extracts from incubations of laurate with liver microsomes and NADPH (a), liver microsomes and $NaIO_4$ (b) and boiled liver microsomes and $NaIO_4$ (c). A 1% OV-17 column was used. Explanation: L = laurate; ω1 = 12-hydroxylaurate; ω2 = 11-hydroxylaurate.

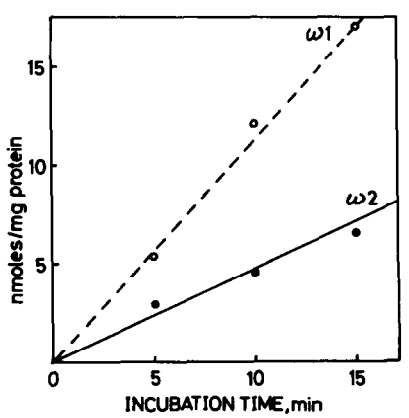

Fig.2. NADPH-supported ω1 and ω2-hydroxylation of laurate as a function of incubation time. Reaction rates are expressed as nmoles of 12-hydroxylaurate (ω1) and 11-hydroxylaurate (ω2) formed per mg of protein per unit of incubation time. For incubation conditions, see Materials and methods.

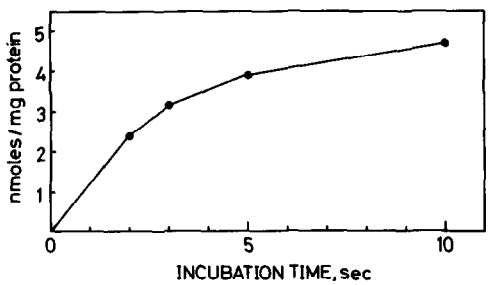

Fig.3. $NaIO_4$-supported ω2-hydroxylation of laurate as a function of incubation time. Reaction rate is expressed as nmoles of 11-hydroxylaurate formed per mg of protein per unit of incubation time. For incubation conditions, see Materials and methods.

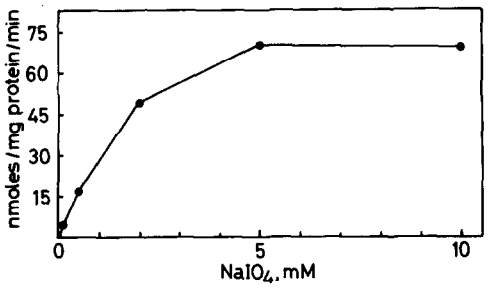

Fig.4. ω2-Hydroxylation of laurate as a function of concentration of $NaIO_4$. Reaction rate is expressed as nmoles of 11-hydroxylaurate formed per mg of protein per min of incubation time. For incubation conditions, see Materials and methods.

As seen in table 1, laurate was the best substrate for microsomal hydroxylase activities, both in NADPH- and in $NaClO_2$- and $NaIO_4$-supported reactions, and therefore laurate was the preferred fatty acid substrate used in the present investigation. It can also be seen that the reaction rates of $NaClO_2$- and $NaIO_4$-supported ω2-hydroxylation of laurate, palmitate and stearate were over 100-fold higher than the rates of the corresponding NADPH-supported hydroxylation reactions. φIO and $\varphi I(OAc)_2$ were even more efficient fortifiers of ω2-hydroxylation of laurate and the rate of the hydroxylation reaction catalyzed by the latter reagent was over 1000-fold higher than the rate of the NADPH-sustained reaction. φIO_2 could not support microsomal hydroxylation of laurate.

Aniline, a compound that produces type II spectral changes when interacting with cytochrome *P*-450, was a potent inhibitor of $NaIO_4$-supported ω2-hydroxylation of laurate. Imidazole, SKF-525A and *p*-aminoglutethimide that are all well-known inhibitors of cytochrome *P*-450 decreased ω2-hydroxylation by about 50% or more. The type II modifier metyrapone and steroids such as progesterone and testosterone were less efficient inhibitors of ω2-hydroxylation. These results indicate an involvement of cytochrome *P*-450 in $NaIO_4$-supported liver microsomal ω2-hydroxylation of laurate. Similar results were obtained with φIO- and $\varphi I(OAc)_2$)-supported ω2-hydroxylation of laurate (data not shown).

4. Discussion

In 1970, Björkhem and Danielsson [15] provided evidence that ω1- and ω2-hydroxylations of fatty

Table 1
ω1- and ω2-Hydroxylation of laurate, palmitate and stearate

Hydroxylating agent	Reaction rates (nmol/mg/min) – Fatty acid substrate					
	Laurate		Palmitate		Stearate	
	ω1	ω2	ω1	ω2	ω1	ω2
NADPH	0.72	0.47	0.34	0.09	0.14	0.03
$NaClO_2$	0[a]	46.0	0	11.9	0	4.50
$NaIO_4$	0	151	0	34.0	0	3.75
φIO	0	188	–[b]	–	–	–
$\varphi I(OAc)_2$	0	930	–	–	–	–
φIO_2	0	0	–	–	–	–

[a] < 0.01
[b] Not analyzed

Table 2
Inhibition of $NaIO_4$-supported ω2-hydroxylation of laurate by modifiers of cytochrome *P*-450

Modifier	Hydroxylation
Control	100
Aniline (5 mM)	0
Imidazole (2 mM)	60
Imidazole (4 mM)	35
SKF-525A (2 mM)	40
p-Aminoglutethimide (50 μg/ml)	54
Metyrapone (0.5 mM)	74
Progesterone (0.2 mM)	74
Testosterone (0.2 mM)	87

Liver microsomes from male rats were incubated with indicated amounts of modifier, laurate and $NaIO_4$ as described in Materials and methods. Values are expressed relative to control.

acids in rat liver microsomes are cytochrome *P*-450-catalyzed reactions, as suggested previously by other workers [16–18]. Björkhem and Danielsson also observed that ω1- and ω2-hydroxylation reactions differed in sensitivity to carbon monoxide and in response to treatment with phenobarbital [15]. The results presented in this paper that $NaClO_2$, $NaIO_4$, φIO and $\varphi I(OAc)_2$ only supported ω2-hydroxylation reactions give further support to the contention that ω1- and ω2-hydroxylations are catalyzed by different species of cytochrome *P*-450 [3], only one of which can be oxygenated by oxidation agents. Furthermore, the results indicate that the active oxygenated intermediate of cytochrome *P*-450 catalyzing ω2-hydroxylation of fatty acids only contains one oxygen atom. This active intermediate may possibly be identical to the ferryl ion complex ($Fe^{4+}\ O^{-}$) possessing a formal oxidation state of +5 and be equivalent to compound I of horse-radish peroxidase, as suggested earlier [4–6,8].

The bypass of the reduction steps involved in NADPH-supported hydroxylation reactions provided by the oxygenating agents resulted in a dramatic increase in the velocity of the hydroxylation reactions. It has been suggested that the rate of reduction of cytochrome *P*-450 is the rate-limiting step in cytochrome *P*-450-catalyzed reactions [19,20]. The present results strongly support this hypothesis and seem to invalidate the conclusion of Hamberg and Björkhem [21], drawn from data on isotope effects involved in 9-hydroxylation of decanoic acid, that the rate-limiting step in ω2-hydroxylation of fatty acids is not reduction of cytochrome *P*-450.

While this work was in manuscript, Lichtenberger et al. reported on iodosobenzene-supported *O*-dealkylation of 7-ethoxycoumarin in rat liver microsomes [22]. Work is now in progress in our laboratories to synthesize various derivatives of φIO and $\varphi I(OAc)_2$ in order to investigate their potency to support cytochrome *P*-450-dependent hydroxylation reactions. Information from this type of work should help in elucidating factors of importance in formation of the active oxygenated intermediate of cytochrome *P*-450.

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