ON THE ABILITY OF CUMENE HYDROPEROXIDE AND $NaIO_4$ TO SUPPORT MICROSOMAL HYDROXYLATIONS IN BIOSYNTHESIS AND METABOLISM OF BILE ACIDS

Henry DANIELSSON and Kjell WIKVALL
Department of Pharmaceutical Biochemistry, University of Uppsala, Box 578, S-751 23 Uppsala, Sweden

Received 21 May 1976

1. Introduction

A number of recent investigations have shown that cumene hydroperoxide, other organic peroxides, $NaIO_4$, and $NaClO_2$ can substitute for NADPH and oxygen in cytochrome *P*-450 dependent hydroxylations in liver microsomes [1–7]. The substrates used in these investigations have been various drugs, steroid hormones and lauric acid. With NADPH and oxygen, lauric acid is hydroxylated in both the 11-position and the 12-position by liver microsomes, whereas with cumene hydroperoxide hydroxylation was obtained only in the 11-position [5]. Ellin and Orrenius [5] concluded that this finding provided further support for the contention that different cytochromes *P*-450 are involved in 11- and 12-hydroxylation of lauric acid. In an extensive study of hydroxylations of steroid hormones in different positions by liver microsomes in the presence of NADPH, cumene hydroperoxide or $NaIO_4$, Hrycay et al. [6,7] found that ratios of products formed from a given steroid differed with the hydroxylating agent. It was suggested that different forms of cytochrome *P*-450 with varying affinity for the hydroxylating agents were involved in the different hydroxylations.

The biosynthesis and metabolism of bile acids include a number of microsomal hydroxylations [8]. These hydroxylations appear all to be cytochrome *P*-450 dependent but differ in one or several respects from each other. The reactions have been shown in reconstituted systems consisting of partially purified cytochrome *P*-450 and NADPH-cytochrome *P*-450 reductase, and attempts are being presently made to study whether or not the different hydroxylase activities correspond to different cytochromes *P*-450 [9]. Information relevant to this question might be obtained from a study of the ability of cumene hydroperoxide or $NaIO_4$ to substitute for NADPH in these hydroxylations.

The present communication reports a comparison between NADPH, cumene hydroperoxide and $NaIO_4$ in their ability to support 6β-, 7α-, 12α-, 25- and 26-hydroxylation in bile acid biosynthesis and metabolism. The results show that of these five NADPH-supported hydroxylations, cumene hydroperoxide supports only 25-hydroxylation and $NaIO_4$ only 25-hydroxylation and to a small but significant extent 6β-hydroxylation.

2. Materials and methods

[1-^{14}C]Lauric acid and [4-^{14}C]cholesterol were obtained from the Radiochemical Centre, Amersham, England. [24-^{14}C]Deoxycholic acid and [24-^{14}C]chenodeoxycholic acid were obtained from ICN, Irvine, Calif., and were coupled with taurine as described by Norman [10]. [^{3}H]Lithocholic acid was prepared by the Wilzbach procedure. 5β-[7β-^{3}H]Cholestane-3α, 7α-diol and 5β-[7β-^{3}H]cholestane-3α, 7α, 12α-triol were prepared by reduction of the corresponding 7-keto compounds [11] with tritium-labeled sodium borohydride (NEN, Dreieichenhain, West Germany). The amount of radioactivity incubated varied between 0.5 and 10 μCi depending on the substrate. NADPH was supplied by Sigma,

St. Louis, Mo. and cumene hydroperoxide by Merck, Darmstadt, West Germany.

Male Sprague-Dawley rats (150–200 g) were used. Liver homogenates (20%, w/v) were prepared in 0.25 M sucrose containing 1 mM EDTA. The microsomal fraction was suspended in 0.1 M potassium phosphate buffer, pH 8.0, in a volume corresponding to that of the original homogenate. The protein content was about 5 mg/ml. The final volume of the incubation mixtures was 5 ml and the concentration of NADPH was 0.7 mM, of cumene hydroperoxide 2 mM or 0.2 mM, and of $NaIO_4$ 10 mM or 1 mM. Incubation mixtures were: lauric acid (50 μg in 25 μl of acetone), 1 ml of microsomes; cholesterol (10 μg in Tween-80), 3 ml of microsomes; 5β-cholestane-3α, 7α-diol and 5β-cholestane-3α, 7α, 12α-triol (250 μg in 50 μl of acetone), 1.5 ml of microsomes; lithocholic acid (50 μg in 50 μl of acetone), taurodeoxycholic acid (200 μg in 100 μl of water) and taurochenodeoxycholic acid (150 μg in 100 μl of water), 1 ml of microsomes.

Incubations were carried out at 37°C for 10 min. Extraction and analytical procedures for the various substrates have been described in several communications from this laboratory [8,11,12]. In these communications, the systems used for thin-layer chromatography are described as well as the method of estimating side-chain hydroxylations of 5β-cholestane-3α, 7α, 12α-triol by radio-gas chromatography. 11- and 12-hydroxylation of lauric acid was analyzed as described by Björkhem and Danielsson [13].

3. Results

Lauric acid, cholesterol, 5β-cholestane-3α, 7α-diol, 5β-cholestane-3α, 7α, 12α-triol, lithocholic acid, taurodeoxycholic acid and taurochenodeoxycholic acid were incubated with rat liver microsomes in the presence of NADPH, cumene hydroperoxide or $NaIO_4$, and the extent of hydroxylation of the different substrates was measured (table 1). 11-Hydroxylation of lauric acid was supported by cumene hydroperoxide in agreement with the findings of Ellin and Orrenius [5] and was also supported by $NaIO_4$. 12-Hydroxylation of lauric acid occurred only with NADPH. In the presence of NADPH, cholesterol was hydroxylated efficiently in the 7α-position and no other products were formed. With cumene hydroperoxide and $NaIO_4$ a number of products were formed. As was expected, the products were those formed from cholesterol by autoxidation, viz. cholestane-3β, 5α, 6β-triol, 7α- and 7β-hydroxycholesterol and 7-ketocholesterol together with some unidentified compounds, probably cholesterol epoxides. With 5β-cholestane-3α, 7α-diol both 12α- and 26-hydroxylation was observed with NADPH, whereas no significant extent of hydroxylation was obtained in the presence of cumene hydroperoxide or $NaIO_4$. 5β-Cholestane-3α, 7α, 12α-triol was hydroxylated primarily in the 25- and 26-positions with NADPH. Hydroxylations in the 23- and 24-positions were also obtained. With cumene hydroperoxide and $NaIO_4$ an efficient 25-hydroxylation was

Table 1
Hydroxylations in the presence of NADPH, cumene hydroperoxide or $NaIO_4$

Reaction	Reaction rates (nmoles/mg protein/10 min)		
	NADPH	Cumene hydroperoxide	$NaIO_4$
11-Hydroxylation of lauric acid	3.4	0.9	3.4
12-Hydroxylation of lauric acid	3.1	< 0.1	< 0.1
12α-Hydroxylation of 5β-cholestane-3α, 7α-diol	0.8	< 0.1	< 0.1
26-Hydroxylation of 5β-cholestane-3α, 7α-diol	1.0	< 0.1	< 0.1
25-Hydroxylation of 5β-cholestane-3α, 7α, 12α-triol	0.6	0.5	0.2
26-Hydroxylation of 5β-cholestane-3α, 7α, 12α-triol	0.7	< 0.1	< 0.1
7α-Hydroxylation of taurodeoxycholic acid	2.7	< 0.1	< 0.1
6β-Hydroxylation of lithocholic acid	2.6	< 0.1	< 0.1
6β-Hydroxylation of taurochenodeoxycholic acid	8.5	< 0.1	< 0.2

obtained, whereas no significant 26-hydroxylation was observed. The bile acids tested were hydroxylated with NADPH in the way described previously, i.e. 7α-hydroxylation of taurodeoxycholic acid and 6β-hydroxylation of lithocholic acid and taurochenodeoxycholic acid. With cumene hydroperoxide no significant hydroxylation of bile acids was observed. With $NaIO_4$ a low extent of 6β-hydroxylation of taurochenodeoxycholic acid could be detected, about 1/40 of that found with NADPH.

The values for different hydroxylations presented in table 1 were obtained with 2 mM cumene hydroperoxide and 10 mM $NaIO_4$. Since it has been shown that these concentrations may inhibit some hydroxylations, the hydroxylations were assayed also with lower concentrations of cumene hydroperoxide, 0.2 mM, and of $NaIO_4$, 1 mM. The extent and pattern of hydroxylations were essentially the same as those given in table 1. Normally, hydroxylations in the presence of NADPH are assayed at pH 7.0–7.4. At pH 8.0 as used in the present investigation, the rates of hydroxylations of the various substrates were up to 25% lower.

4. Discussion

Several lines of evidence indicate that many of the hydroxylations occurring in the biosynthesis and metabolism of bile acids are specific and in one or several respects different from other microsomal hydroxylations occurring with fatty acids, steroid hormones and foreign compounds [8,9,11,12]. In this respect, the 7α-hydroxylation of cholesterol, the 12α-hydroxylation and the 26-hydroxylation are particularly noteworthy. These three hydroxylations seem to play important roles in regulation of overall bile acid biosynthesis or of synthesis of individual bile acids. So far, it has not been possible to ascribe these hydroxylations to specific species of cytochrome *P*-450. The results of the present investigation can be considered to provide further evidence for the specificity of some of the hydroxylations involved in the biosynthesis and metabolism of bile acids. The inability of cumene hydroperoxide and $NaIO_4$ to support what appears to be especially important reactions in bile acid biosynthesis is striking. Whereas a rather efficient 25-hydroxylation of 5β-cholestane-3α, 7α, 12α-triol was obtained with these agents, no 26-hydroxylation was observed. At least in the rat, 26-hydroxylation is an important step in the oxidation of the C_{27} side chain, whereas 25-hydroxylation appears to be a side reaction. The 25-hydroxylation of 5β-cholestane-3α, 7α, 12α-triol resembles in several respects hydroxylations occurring with different drugs and other foreign compounds in liver microsomes. It is tempting to suggest that cumene hydroperoxide and $NaIO_4$ primarily will support more unspecific 'drug hydroxylations'. In consonance with this contention, cumene hydroperoxide and $NaIO_4$ support 11- but not 12-hydroxylation of lauric acid. Whereas microsomal ω-hydroxylation is the first step in the ω-oxidation of fatty acids, no specific biological role has yet been ascribed to (ω−1)-hydroxylation. With respect to hydroxylations of steroid hormones by liver microsomes, which are supported by cumene hydroperoxide and $NaIO_4$ [6,7], it can not be stated that these reactions are not important under physiological conditions. However, they resemble in many respects drug hydroxylations.

From their studies of hydroxylations of lauric acid and steroid hormones Ellin and Orrenius [5] and Hrycay et al. [7] have concluded that the differences in pattern of hydroxylations with NADPH as compared with cumene hydroperoxide and $NaIO_4$ can be ascribed to different species of cytochrome *P*-450 catalyzing different hydroxylations. The present results are not at variance with this contention.

Acknowledgements

The skillful technical assistance of Mrs Britt-Marie Johansson is gratefully acknowledged. This study was supported by a grant from the Swedish Medical Research Council (project No., 03X-218).

References

[1] Kadlubar, F. F., Morton, K. C. and Ziegler, D. M. (1973) Biochem. Biophys. Res. Commun. 54, 1255–1261.
[2] Rahimtula, A. D. and O'Brien, P. J. (1974) Biochem. Biophys. Res. Commun. 60, 440–447.
[3] Rahimtula, A. D. and O'Brien, P. J. (1975) Biochem. Biophys. Res. Commun. 62, 268–275.

[4] Burke, M. D. and Mayer, R. T. (1975) Drug Metabolism and Disposition 3, 245–253.
[5] Ellin, A. and Orrenius, S. (1975) FEBS Lett. 50, 378–381.
[6] Hrycay, E. G., Gustafsson, J.-Å., Ingelman-Sundberg, M. and Ernster, L. (1975) FEBS Lett. 56, 161–165.
[7] Hrycay, E. G., Gustafsson, J.-Å., Ingelman-Sundberg, M. and Ernster, L. (1976) Eur. J. Biochem. 61, 43–52.
[8] Björkhem, I. and Danielsson, H. (1974) Mol. Cell. Biochem. 4, 79–95.
[9] Björkhem, I., Danielsson, H. and Wikvall, K. (1975) Biochem. Soc. Trans. 3, 825–828.
[10] Norman, A. (1955) Ark. Kemi 8, 331–342.
[11] Björkhem, I., Danielsson, H. and Wikvall, K. (1976) J. Biol. Chem. 251, in press.
[12] Björkhem, I., Danielsson, H. and Wikvall, K. (1974) J. Biol. Chem. 249, 6439–6445.
[13] Björkhem, I. and Danielsson, H. (1970) Eur. J. Biochem. 17, 450–459.