THE ELECTRON TRANSPORT MECHANISM ASSOCIATED WITH 12α-HYDROXYLATION OF C_{27} STEROIDS

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The biosynthesis of cholic acid from cholesterol requires the introduction of a 12α-hydroxyl group. The substrate for 12α-hydroxylation is probably 7α-hydroxycholest-4-en-3-one (7α-HC), a compound that is converted efficiently into 7α,12α-dihydroxycholest-4-en-3-one (7α,12α-DHC) by rat-liver microsomes (Danielsson and Einarsson, 1966). The latter compound is easily reduced to 3α,7α,12α-trihydroxy-5β-cholestane (Berséus et al., 1965), a precursor of cholic acid (Danielsson, 1963).

Liver microsomes hydroxylate various drugs in the presence of O_2 and NADPH. The O_2 used in these hydroxylations is activated by cytochrome P-450, a haemoprotein which, in the reduced state, combines with CO (Estabrook et al., 1963). According to the current view (see Mason et al., 1965; Sato et al., 1965; Omura et al., 1965), P-450 acts as the terminal component of an electron-transfer chain containing as the primary enzyme an NADPH-specific flavoprotein with cytochrome c reductase activity:-

NADPH → NADPH-specific flavoprotein → unknown factor → P-450 (1)

A similar electron chain has been shown to operate in the hydroxylations of steroids by adrenal cortex mitochondria (Omura et al., 1966). Omura and Sato (1964) have described another microsomal electron-transfer chain which uses cytochrome b_5, but no physiological functions have yet been assigned to this. Finally, Oshino et al. (1966) have shown that the oxidative

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desaturation of fatty acids brought about by liver microsomes requires a cyanide-sensitive factor.

In this paper we describe experiments on the co-factor requirements of the 12α-hydroxylation of 7α-HC by rat-liver microsomes. Our experiments show that neither P-450 nor the cyanide-sensitive factor of Oshino et al. (1966) is required for this hydroxylation.

METHODS

For each experiment, the livers of 2-3 rats were homogenized with 4 vol. of cold 0.25 M sucrose. The supernatant obtained by spinning the homogenate at 1.2×10^4 x g. min. was centrifuged at 1.65×10^5 x g. min. and the microsomes were prepared from the supernatant from this spin by centrifugation at 6.2×10^6 x g. min. The microsomal pellet was washed three times with cold 0.25 M sucrose and suspended in this medium, 1 ml. containing the microsomes from 1 g. wet liver. The microsomes were contaminated with mitochondria to the extent of 1-2 %, as estimated from the specific activity of succinate-cytochrome c reductase (Green et al., 1955) in microsomes and mitochondria. The incubation mixture, extractions and analysis of the extracts by thin-layer chromatography were essentially those described by Danielsson and Einarsson (1966). Phenobarbital was given intraperitoneally (100 mg./kg. body weight) every other day for five days, and the rats were killed seven days after the first injection. Cytochromes b_5 and P-450 were estimated essentially by the method of Omura and Sato (1964). NADPH-cytochrome c reductase activity was assayed by the method of Phillips and Langdon (1962). The ^{3}H-7α-HC and markers of 7α-HC and 7α,12α-DHC were a generous gift from Dr. Henry Danielsson of the Karolinska Institutet, Stockholm.

RESULTS AND DISCUSSION

In confirmation of previous work (Danielsson and Einarsson, 1966), when ^{3}H-7α-HC was incubated for 10 min. with rat-liver microsomes in the presence of NADPH, about 20 % of the substrate was converted into 7α,12α-

DHC. NADPH was necessary for the hydroxylation and could not be replaced by NADH or ascorbate (Table 3). Both NADH and ascorbate are efficient electron donors for the oxidative desaturation of the CoA esters of fatty acids by liver microsomes although they cannot replace NADPH in the microsomal hydroxylation of drugs (Oshino et al., 1966).

A requirement for molecular oxygen for the 12α-hydroxylation reaction is shown in Table 1. Little or no reaction was observed when N_2 replaced a mixture of N_2:O_2 (9:1, v/v) as the gas phase. CO had no effect on the hydroxylation of 7α-HC, even with CO:O_2 ratios as high as 9:1. When the standard incubation mixture was gassed with this gas mixture, 100 % of the microsomal P-450 was shown by spectrophotometric measurement to be in the form of the CO complex. The microsomal 12α-hydroxylation of 7α-HC therefore differs from hydroxylations involving P-450, since these are inhibited by CO (Omura et al., 1965).

TABLE 1

Requirement for oxygen and effect of carbon monoxide on 12α-hydroxylation of 7α-HC by rat-liver microsomes

Gas mixture	% of added ^3H recovered as 7α,12α-DHC	% of added ^3H recovered as 7α-HC	Relative activity
N_2:O_2 (9:1, v/v)	31.7	52.2	100.0
N_2 (100 %)	4.5	81.1	14.5
CO:O_2 (9:1, v/v)	41.5	44.1	133.2

The standard incubation mixture contained 0.6 ml. of microsomal suspension; K phosphate buffer, pH 7.5 (76 mM); $MgCl_2$ (3.8 mM); nicotinamide (23 mM); NADPH (1 mM); ^3H-7α-HC (12 μM, with 0.3 μC); final volume 3.15 ml. Duplicate incubations were carried out for 10 min. at 37°C.

Ernster and Orrenius (1965) have suggested that P-450 is rate-limiting for microsomal hydroxylations, since drug-hydroxylating activity and the P-450 content of liver microsomes increase in parallel when rats are treated with phenobarbital. We have therefore tested the effect of phenobarbital treatment on the 12α-hydroxylation of 7α-HC. In confirmation of previous work (Orrenius et al., 1965), the liver microsomes of the treated rats

showed an increased content of P-450 and cytochrome b_5 and an increased activity of NADPH-cytochrome c reductase (Table 2). The 12α-hydroxylating

TABLE 2

Effect of phenobarbital on the liver microsomal activity of 12α-hydroxylase and NADPH-cytochrome c reductase, and on the liver microsomal content of P-450 and cytochrome b_5

Preparation	Specific activity*			
	12α-Hydroxylase	P-450	b_5	NADPH-cytochrome c reductase
Control	0.491	0.810	0.452	148
Phenobarbital-treated	0.095	2.260	0.635	247
Control + phenobarbital (10 μg./flask)	0.561			
Control + phenobarbital (50 μg./flask)	0.550			

Incubation mixture as in Table 1.

* P-450 and b_5 contents are expressed in mμmoles/mg. protein; 12α-hydroxylase activity is expressed in mμmoles of 7α,12α-DHC formed per 10 min. per mg. protein; NADPH-cytochrome c reductase activity is expressed in mμmoles of cytochrome c reduced per min. per mg. protein.

activity, however, was depressed. This depression was not due to competitive inhibition by phenobarbital present in the microsomal preparation, since phenobarbital added to the incubation mixture did not inhibit 12α-hydroxylation.

Table 3 shows the effect of various other reagents on NADPH-dependent 12α-hydroxylation. KCN did not inhibit 12α-hydroxylation, showing that this reaction does not require the cyanide-sensitive factor of Oshino et al. (1966). Cytochrome c depressed 12α-hydroxylation to about a third of the control value. This effect is to be expected since the NADPH-specific flavoprotein possesses cytochrome c reductase activity. In the presence of cytochrome c oxidase due to mitochondrial contamination (see Methods), cytochrome c would therefore tend to cause a leak of electrons from NADPH and would thus diminish the reducing power available for the hydroxylation

TABLE 3

The effect of electron donors and various inhibitors on the 12α-hydroxylation of 7α-HC by rat-liver microsomes

Modification to standard incubation	% of added ^3H recovered as 7α,12α-DHC	% of added ^3H recovered as 7α-HC	Relative activity
Complete system	18.1	71.5	100.0
No NADPH	1.5	87.5	8.2
No NADPH, + NADH (1 mM)	3.2	86.7	17.5
No NADPH, + ascorbate (10 mM)	1.5	87.1	8.2
KCN (1 mM)	21.2	66.6	117.1
Cytochrome c (0.01 mM)	6.1	86.6	33.7
Quinacrine (1 mM)	12.5	80.7	69.1
α,α'-Dipyridyl (1 mM)	24.9	66.2	137.6
o-Phenanthroline (1 mM)	14.1	75.3	77.9
Catalase (1 mg./flask)	16.5	72.8	91.2

Incubation mixture as in Table 1.

reaction. 12α-Hydroxylating activity was slightly depressed by quinacrine, a flavoprotein inhibitor (Ryan and Engel, 1957), but was not consistently affected by the two chelating agents, o-phenanthroline and α,α'-dipyridyl. Catalase had no effect on 12α-hydroxylation, indicating that H_2O_2 does not participate in the reaction.

These experiments show that the 12α-hydroxylase concerned in the formation of cholic acid requires NADPH and O_2, in this respect resembling the "mixed function" oxidases concerned in the hydroxylations of drugs and steroids by liver and adrenal cortex. The lack of inhibitory effect of CO and the lack of correlation between microsomal P-450 and 12α-hydroxylating activity in the phenobarbital-treated rats suggest, however, that P-450 is not the terminal oxidase in 12α-hydroxylation. The inhibitory effect of cytochrome c is consistent with the possibility that, in the

12α-hydroxylation of 7α-HC, the electron acceptor from NADPH is the NADPH-specific flavoprotein (reaction sequence 1) that participates in microsomal drug hydroxylations and in the oxidative desaturation of fatty acids.

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