HETEROGENEITY OF HEPATIC MIXED FUNCTION OXIDASES

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SUMMARY

The effects of NADH and increasing concentrations of potassium phosphate buffer, potassium chloride and potassium thiocyanate on several hydroxylations catalyzed by rat liver microsomes were studied. All the hydroxylations were stimulated by NADH in the presence of suboptimal concentrations of NADPH. The 7α-hydroxylation of cholesterol, the 12α-hydroxylation of 7α-hydroxy-4-cholesten-3-one and the 16-hydroxylation of palmitic acid were inhibited by increasing concentrations of potassium phosphate buffer, potassium chloride and potassium thiocyanate to a greater extent than any of the other hydroxylations studied. This finding and the previous finding that these three hydroxylations are not stimulated by phenobarbital treatment suggest differences between these hydroxylations and most other microsomal hydroxylations in liver. The possibility is discussed that different types of cytochrome P-450 may be involved.

Most microsomal hydroxylations of drugs, steroids and fatty acids in liver share the common characteristics of being inhibited by carbon monoxide and stimulated by phenobarbital (1). Carbon monoxide reduces the amount of active cytochrome P-450 available and phenobarbital increases the amounts of cytochrome P-450 and NADPH-cytochrome P-450 reductase in the liver (1). Three microsomal hydroxylations in rat liver have been reported to be sensitive to carbon monoxide but unaffected or inhibited by phenobarbital, at least in Sprague-Dawley rats, the 7α-hydroxylation of cholesterol, the 12α-hydroxylation of 7α-hydroxy-4-cholesten-3-one and the ω1-hydroxylation of long-chain fatty acids (2,3). The reason for the difference between these three hydroxylations and other microsomal hydroxylations is not known. Different types of cytochrome P-450 or different types of NADPH-cytochrome P-450 reductase might be involved or the concentration of one or both of the two factors might not

be rate limiting under the experimental conditions.

It was considered of interest to study whether the three hydroxylations discussed above differ from other microsomal hydroxylations in more ways than their reaction to phenobarbital treatment. The present work reports studies of the effects of NADH and potassium phosphate, potassium chloride and potassium thiocyanate on several microsomal hydroxylations including the 7α-hydroxylation of cholesterol, the 12α-hydroxylation of 7α-hydroxy-4-cholesten-3-one and the 16-hydroxylation of palmitic acid. The effect of NADH was studied because of the recent finding that NADH stimulates microsomal drug hydroxylation at suboptimal concentrations of NADPH (4). The effect of the inorganic ions was studied because of the findings that potassium phosphate buffer stimulates NADPH-cytochrome P-450 reductase activity (5) and that active cytochrome P-450 is converted into inactive cytochrome P-420 in the presence of solutions of high ionic strength (6).

EXPERIMENTAL PROCEDURE

Materials. 4-^{14}C-Dehydroepiandrosterone (3β-hydroxy-5-androsten-17-one), 4-^{14}C-testosterone, 4-^{14}C-cholesterol, and 1-^{14}C-palmitic acid were obtained from Radiochemical Centre (Amersham, England) and had specific radioactivities of 0.14, 0.29, 59 and 40 μC/μmole, respectively. Tritium-labeled taurodeoxycholic acid and taurochenodeoxycholic acid (specific radioactivities 2.6 and 3.5 μC/μmole, respectively) were synthesized from tritium-labeled deoxycholic acid and chenodeoxycholic acid as described by Norman (7). 6β-^{3}H-7α-Hydroxy-4-cholesten-3-one was synthesized as described previously (8) and had a specific radioactivity of 3.2 μC/μmole.

Methods. White male rats of the Sprague-Dawley strain, weigh-

ing 150-200 g, were used. Liver homogenates, 20% (w/v), were prepared in 0.1 M potassium phosphate buffer, pH 7.4, and the microsomal fraction was isolated as described previously (9,10). In the case of preparation of microsomes designed for incubations with cholesterol, a 0.25 M sucrose solution containing 1 mM EDTA was used as homogenizing medium in order to avoid lipid peroxidation (10). The microsomal pellet was resuspended in 0.1-1.0 M potassium phosphate buffer or in 0.1 M potassium phosphate buffer, pH 7.4, containing 0.05-0.4 M potassium thiocyanate. In the experiments with varying concentrations of potassium chloride, 0.03 M potassium phosphate buffer, pH 7.4, was added to buffer the solution. The protein concentration of the microsomal fraction was about 4 mg/ml when determined with the Lowry method (11). Incubations with tritium-labeled taurodeoxycholic acid and taurochenodeoxycholic acid were performed as described by Einarsson and Johansson (9) with the exception that 3 μmoles of NADPH were used instead of an NADPH-generating system. The incubations were terminated by the addition of ethanol and were then saponified and extracted with ether as described previously (9). The ether extract was treated with diazomethane and subjected to radio-gas chromatography using a Barber-Colman 5000 instrument equipped with a 3% QF-1 column. The extent of conversion was calculated from the radioactivity tracing. Incubations with 4-^{14}C-dehydroepiandrosterone, 4-^{14}C-testosterone, 4-^{14}C-cholesterol, and 6β-^{3}H-7α-hydroxy-4-cholesten-3-one as well as subsequent analyses by thin-layer chromatography were performed as described previously (8,10,12). Incubations with 1-^{14}C-palmitic acid and subsequent analysis with radio-gas chromatography were performed as described previously (3). Incubations with aminopyrine and

Table 1. Effect of NADH on microsomal hydroxylation of different steroids and palmitic acid in the presence of suboptimal concentrations of NADPH

Reaction	NADPH 0.1 μmoles	NADPH 0.1 μmoles + NADH 1 μmole	NADPH 0.5 μmoles	NADPH 0.5 μmoles + NADH 1 μmole	NADPH 3 μmoles	NADPH 3 μmoles + NADH 1 μmole
7α-Hydroxylation of dehydroepiandrosterone	3.1	8.4	10.8	16.6		
6β-Hydroxylation of testosterone	1.6	5.2	5.6	10.5	17.0	21.1
7α-Hydroxylation of taurodeoxycholic acid	2.7	5.6	5.4	12.5	16.3	18.6
12α-Hydroxylation of 7α-hydroxy-4-cholesten-3-one	0.7	1.6	2.8	4.6	7.8	9.9
7α-Hydroxylation of cholesterol	0.2	0.9	0.9	1.5	1.4	1.8
16-Hydroxylation of Palmitic acid	0.8	2.0	4.1	10.5	8.4	13.7

subsequent analysis were performed as described by Einarsson and Johansson (13).

RESULTS

Effect of NADH at suboptimal concentrations of NADPH. Table 1 summarizes the effect of NADH on microsomal hydroxylation of different steroids and palmitic acid in the presence of suboptimal concentrations of NADPH. In all cases, NADH stimulated the reaction and the extent of stimulation was highest at low concentrations of NADPH.

Effect of potassium phosphate buffer, potassium chloride and potassium thiocyanate. Fig. 1 shows the experiments with increasing concentrations of potassium phosphate buffer. The 7α-

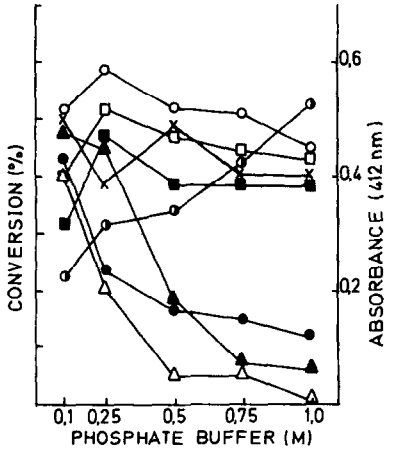
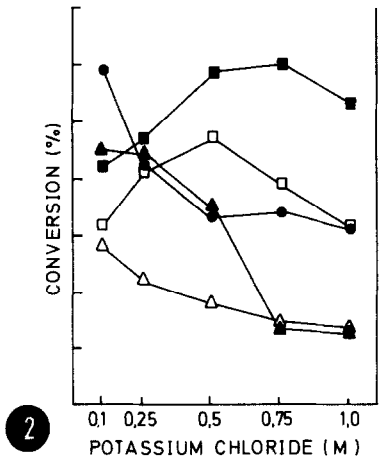


Fig. 1. Effect of potassium phosphate buffer on 7α-hydroxylation of dehydroepiandrosterone ■—■, 6β-hydroxylation of testosterone □—□, 6β-hydroxylation of taurochenodeoxycholic acid O—O, 7α-hydroxylation of taurodeoxycholic acid X—X, 12α-hydroxylation of 7α-hydroxy-4-cholesten-3-one ●—●, 7α-hydroxylation of cholesterol △—△, 16-hydroxylation of palmitic acid ▲—▲, and oxidative demethylation of aminopyrine ◑—◑. The scale on the left axis represents 0-0.7% of added substrate in the case of 7α-hydroxylation of cholesterol, 0-7% in the case of 12α-hydroxylation of 7α-hydroxy-4-cholesten-3-one and 16-hydroxylation of palmitic acid, and 0-14% in the case of 7α-hydroxylation of dehydroepiandrosterone and taurodeoxycholic acid, 6β-hydroxylation of testosterone and 6β-hydroxylation of taurochenodeoxycholic acid. The scale on the right axis represents the absorbance at 412 nm obtained in the spectrophotometric assay of oxidative demethylation of aminopyrine (cf. Methods).

hydroxylation of cholesterol, the 12α-hydroxylation of 7α-hydroxy-4-cholesten-3-one and the 16-hydroxylation of palmitic acid were inhibited whereas all the other hydroxylations were unaffected or stimulated. Potassium chloride also inhibited the three hydroxylations (Fig. 2) although to a less extent than potassium phosphate, indicating that the effect of potassium phosphate may depend on both potassium and phosphate ions. Fig. 3 shows the experiments with different concentrations of potassium thiocyanate. The 7α-hydroxylation of cholesterol, the 12α-hydroxylation of 7α-hydroxy-4-cholesten-3-one



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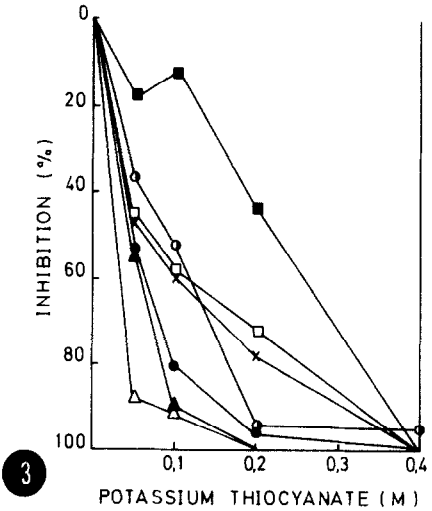

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Fig. 2. Effect of potassium chloride. Scales and symbols as in Fig. 1.
Fig. 3. Effect of potassium thiocyanate. Symbols as in Fig. 1.

and the 16-hydroxylation of palmitic acid were inhibited at lower concentrations of potassium thiocyanate than the other hydroxylations. All the hydroxylations were completely inhibited in the presence of 0.4 M potassium thiocyanate.

DISCUSSION

The present work shows that the stimulation of microsomal

hydroxylations by NADH is not restricted to hydroxylations of drugs as hydroxylation of the different steroids and palmitic acid was stimulated in a similar way. It appears unlikely that there are fundamental differences with respect to the mechanism of reduction of the cytochrome P-450-substrate complex between the different hydroxylations studied in the present investigation. The selective inhibition by potassium phosphate buffer of 7α-hydroxylation of cholesterol, 12α-hydroxylation of 7α-hydrocy-4-cholesten-3-one and 16-hydroxylation of palmitic acid indicates a distinct difference between these hydroxylations and the other hydroxylations. It seems less likely that this inhibitory effect is due to an effect on the cytochrome P-450 reductase system as it has been previously shown that potassium phosphate buffer up to a concentration of 0.8 M stimulates NADPH-cytochrome P-450 reductase activity (5). It might be argued that a specific type of NADPH-cytochrome P-450 reductase is involved in the three hydroxylations with properties different from the bulk of NADPH-cytochrome P-450 reductase in liver microsomes. At least in the case of the 7α-hydroxylation of cholesterol this seems less likely in view of the finding that an antibody against NADPH-cytochrome P-450 reductase inhibits the reaction (14). A more probable explanation for the effect of potassium phosphate buffer on the hydroxylations may be that the potassium phosphate interacts with the cytochrome P-450 involved in the hydroxylation. It is known that high concentrations of different buffers and neutral salts convert active cytochrome P-450 into inactive cytochrome P-420 (6). In the case of potassium phosphate it has been reported that the bulk of cytochrome P-450 is not converted into cytochrome P-420 in the presence of potassium phosphate buffer of

lower concentrations than 2 M (6). This does not exclude the possibility that a specific type of cytochrome P-450 might be converted into cytochrome P-420 at a considerably lower concentration of potassium phosphate buffer. It has been reported that microsomal cytochrome P-450 is converted quantitatively into cytochrome P-420 in the presence of 0.4 M potassium thiocyanate (6). In agreement with this finding, all the hydroxylations studied in the present work were completely inhibited in the presence of 0.4 M potassium thiocyanate. The fact that the cholesterol 7α-hydroxylase, the 7α-hydroxy-4-cholesten-3-one 12α-hydroxylase and the palmitic acid 16-hydroxylase were more sensitive to potassium thiocyanate makes it tempting to suggest that the cytochrome(s) P-450 involved in these hydroxylations differs from the bulk of cytochrome P-450 in the microsomes. This contention is also supported by the previous finding that these three hydroxylations in contrast to most other hydroxylations are not stimulated by treatment with phenobarbital. It may also be mentioned that biliary drainage (2) and adjuvant-induced arthritis (15) have been shown to lead to a decrease of the bulk of cytochrome P-450 in the liver but to an increased or unchanged cholesterol 7α-hydroxylase activity.

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