ON THE MECHANISM OF 11-HYDROXYLATION OF LAURIC ACID BY RAT LIVER MICROSOMES

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SUMMARY

11-$^{2}H_2$-Lauric acid with a randomly distributed tritium label was synthesized and the rate of 11-hydroxylation of this compound by rat liver microsomes was compared with the rate of 11-hydroxylation of 1-^{14}C-lauric acid. The 11-$^{2}H_2$-label was found to decrease the V_{max} of the reaction with a factor of about 3.6 and the K_m with a factor of about 1.5. The results support the previous suggestion that the rate-limiting step in microsomal 11-hydroxylation of lauric acid is the rate of cleavage of the C-H bond.

It has been recently shown that microsomal hydroxylation of some specifically deuterium-lateled steroids and fatty acids leads to a kinetic isotope effect (1-4). The isotope effect has been of such an order of magnitude (>2) to make it probable that cleavage of the C-^{2}H bond occurs in a rate-limiting step in the reaction. The technique used in these experiments has been to mix the deuterated compound with the corresponding unlabeled compound and analyze the ratio between the product formed from the deuterated compound and that formed from the unlabeled compound. Isotopes in stable positions have been used to mark the deuterated and/or the unlabeled compound. The apparent isotope effect estimated in these experiments might not be fully accurate due to the possibility that there is an inhibition of the hydroxylation of the deuterated molecules by the unlabeled molecules or vice versa. It would be preferable to compare the rate of hydroxylation of the deuterated compound with the rate of hydroxyla-

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tion of the undeuterated compound in separate experiments. It has not been possible to perform such experiments with the hydroxylations so far studied since the rate of reaction has been too slow to permit accurate assays. Recently it was reported (4) that 11-D- and 11-L-hydroxylation of 11-$^{2}H_{2}$-lauric acid by the microsomal fraction of rat liver was accompanied by a marked isotope effect. As microsomal 11-hydroxylation of lauric acid is very efficient it should be possible to determine the isotope effect in the 11-hydroxylation of 11-$^{2}H_{2}$-lauric acid as outlined above. Furthermore, the technique would permit determination of the effect of deuterium substitution on V_{max} and K_{m} which should provide information concerning the nature of the isotope effect.

MATERIALS AND METHODS

1-^{14}C-Lauric acid (20 μC/mg) was obtained from the Radiochemical Centre and was purified with silicic acid chromatography (4). 11-$^{2}H_{2}$-Lauric acid was prepared as described previously (4). The material was tritiated according to Wilzbach (5) by exposure to ^{3}H gas (3 C) for two weeks. The material was then refluxed with 2 N potassium hydroxide in methanol for 24 hours and was purified by silicic acid chromatography. Combined gas chromatography-mass spectrometry showed that the material consisted to more than 96% of dideuterated molecules showing that there had been no loss of deuterium during the Wilzbach procedure. The specific radioactivity was 0.85 mC/mg and as expected no significant amount of tritium could be detected by mass spectrometry. The procedure for preparation of the microsomal preparation was as described previously (4). Lauric acid, 50 μg dissolved in 50 μl of acetone, was added to 1 ml of microsomal suspension (containing about 5 mg protein

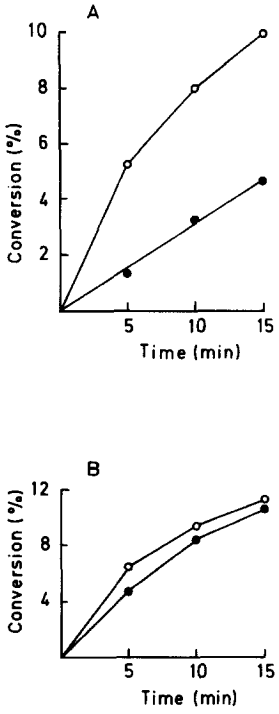

Fig. 1. Effect of time on (A) 11-hydroxylation and (B) 12-hydroxylation of 1-^{14}C-lauric acid (O—O) and tritium labeled 11-$^{2}H_2$-lauric acid (●—●).

per ml) together with an NADPH-generating system (6) in a total volume of 3 ml of a modified Bucher medium (7). The extracts of the incubations were treated with diazomethane and the amounts of ω1- and ω2-hydroxylated products were assayed with radio-gas chromatography (4). No attempt was made to separate the 11-D- and 11-L-isomers of 11-hydroxy-lauric acid (cf. (4)).

RESULTS

Fig. 1 shows the effect of time on 11- and 12-hydroxylation of 11-$^{2}H_2$- and 1-^{14}C-lauric acid. No isotope effect was observed in the 12-hydroxylation of 11-$^{2}H_2$-lauric acid, whereas an isotope effect varying between 2.2 and 3.5 was observed in the 11-hydroxylation of 11-$^{2}H_2$-lauric acid. The most marked

Table I

Experiment with mixture of 1-^{14}C- and 11-$^{2}H_2$-lauric acids

Compound	^{3}H	^{14}C	$^{3}H/^{14}C$
	c.p.m.	c.p.m.	
1-^{14}C-lauric acid + tritium labeled 11-$^{2}H_2$-lauric acid incubated	12,444	1,136	11.0
11-Hydroxylauric acid isolated	2,014	388	5.2
12-Hydroxylauric acid isolated	9,552	766	12.4

isotope effect was seen in the early phase of the time curve. The isotope effect in the 11-hydroxylation of 11-$^{2}H_2$-lauric acid was further shown by conversion of a mixture of 11-$^{2}H_2$-lauric acid and 1-^{14}C-lauric acid into 11- and 12-hydroxylauric acid. The 11- and 12-hydroxylauric acids were isolated by thin-layer chromatography (3) and the $^{3}H/^{14}C$ ratio in the 11-hydroxylauric acid was found to be about half that of the starting material (Table I). The 12-hydroxylauric acid isolated had a $^{3}H/^{14}C$ ratio slightly higher than the starting material. In Fig. 2 a Lineweaver-Burk plot of 1/V and 1/S for the 11-hydroxylation of the two substrates is shown. K_m for 1-^{14}C-lauric acid was about 17 μM and for 11-$^{2}H_2$-lauric acid about 11 μM. The ratio between V_{max} for the 11-hydroxylation of 1-^{14}C-lauric acid and for the 11-hydroxylation of 11-$^{2}H_2$-lauric acid was about 3.6.

DISCUSSION

In the previous report (4) it was shown that microsomal 11-L- and 11-D-hydroxylation of a mixture of 11-$^{2}H_2$-lauric acid and 11-$^{1}H_2$-lauric acid yielded 11-hydroxylauric acid with a ratio $\frac{\text{deuterated molecule}}{\text{undeuterated molecule}}$ that was only about 1/3 of

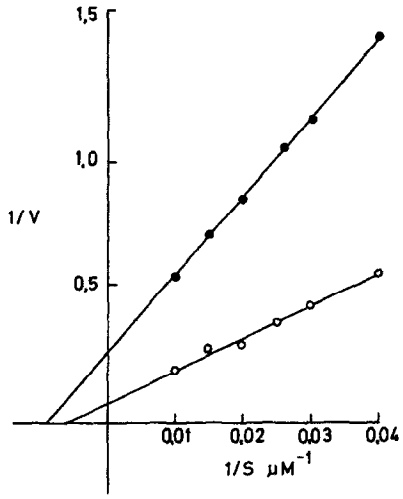

Fig. 2. Lineweaver-Burk plot of 1/V and 1/S from experiments with 1-^{14}C-lauric acid and tritium labeled 11-$^{2}H_2$-lauric acid. Symbols as in Fig. 1. V corresponds to μg 11-hydroxylauric acid formed under the conditions of the assay.

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the corresponding ratio in the starting material. In the present work, where the rates of 11-hydroxylation of 11-$^{2}H_2$-lauric acid (with tritium label as marker) and of 11-$^{1}H_2$-lauric acid (with 1-^{14}C label as marker) were determined independently, the isotope effect was about the same. A somewhat lower isotope effect was observed for 11-hydroxylation of a mixture of tritium-labeled 11-$^{2}H_2$-lauric acid and 1-^{14}C-lauric acid. It is conceivable that the deuterated molecules hinder hydroxylation of undeuterated molecules which would lead to an erroneously low figure for the isotope effect. The 12-hydroxylauric acid isolated from the experiments with the mixture of tritium-labeled 11-$^{2}H_2$-lauric acid and 1-^{14}C-lauric acid had a slightly higher $^{3}H/^{14}C$ ratio than the starting material, indicating a slightly faster rate of 12-hydroxylation of 11-$^{2}H_2$-lauric acid than of 1-^{14}C-lauric acid. However, no such effect was observed in the experiments where the rate of 11-

and 12-hydroxylation of 11-$^{2}H_{2}$-lauric acid and 1-^{14}C-lauric acid were assayed independently.

The isotope effect can be explained in two ways; cleavage of the C-H bond is rate limiting in 11-hydroxylation or the enzyme has a much lower affinity for 11-$^{2}H_{2}$-lauric acid than for 1-^{14}C-lauric acid. In the latter case a smaller isotope effect than that observed would be expected. Furthermore, a decreased binding of 11-$^{2}H_{2}$-lauric acid to the enzyme should result in a higher K_m-value. In contrast, a lower K_m-value was observed for hydroxylation of 11-$^{2}H_{2}$-lauric acid than for 1-^{14}C-lauric acid indicating a stronger binding of the deuterated compound to the enzyme and/or a decreased rate of breakdown of the enzyme-substrate complex. A decrease in rate of breakdown of the enzyme-substrate complex is to be expected if cleavage of the C-H bond is rate limiting. The present results thus provide further evidence that the rate-limiting step in the 11-hydroxylation of lauric acid is cleavage of the C-H bond. That microsomal hydroxylation of a compound which involves cleavage of the C-H bond as a rate-limiting step should be characterized by an isotope effect above 2 and a decreased K_m value with a specifically deuterated substrate has been previously emphasized by Mitoma *et al.* (8) and Elison *et al.* (9). Mitoma *et al.* found that microsomal demethylation of o-nitroanisol with an O-C$^{2}H_{3}$ group was accompanied with an isotope effect varying between 1.5 and 2.8 and that the deuterium label decreased the K_m for the reaction by about 50%. It was concluded that microsomal demethylation of o-nitroanisol involves cleavage of the C-H bond as a rate-limiting step. Elison et al. (9) found that demethylation of morphine with an N-C$^{2}H_{3}$ group occurred with an isotope effect of about 1.4

and that the deuterium label increased the K_m value. In this case, it was concluded that demethylation of morphine does not include cleavage of the C-H bond as a rate-limiting step.

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