2-HYDROXY FATTY ACID OXIDASES OF RAT KIDNEY

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SUMMARY: The 104000xg supernatant of homogenates from rat kidney catalyses the oxidation of DL,2-hydroxystearate to the corresponding 2-keto acid which is decarboxylated in the microsomal fraction. Two 2-hydroxy fatty acid oxidases, specific for the D and L isomers respectively, have been separated. The activity of these partially purified oxidases decreases in the absence of NAD^+.

INTRODUCTION: The 2-hydroxy fatty acids are components of the glycolipids of most mammalian tissues (1), and have been identified as intermediates in the a-oxidation of the long-chain fatty acids (2,3). Ceramides, the product of partial degradation of glycolipids, are enzymatically hydrolyzed to sphingosine and fatty acids (4). An enzymatic system which decarboxylates the 2-hydroxy fatty acids has been identified in brain microsomes (5,6,7). This report describes the separation from non-nervous tissues of two soluble 2-hydroxy fatty acid oxidases, specific for the conversion of the D and L isomers, to the corresponding 2-keto acid. This latter is decarboxylated in the microsomal fraction.

MATERIAL AND METHODS: The DL,2-hydroxystearic acid-1-C^{14} and the methyl ester of the 2-ketostearic acid were prepared as described previously (5). To prepare the 2,4-dinitrophenylhydrazone, 50mg of the 2-keto methyl ester were allowed to react for 5 min., at room temperature, with 2.5ml of a freshly prepared solution of 2,4-dinitrophenylhydrazine (5% in methanol-sulfuric acid 9:1 v/v). The lipid material was taken up into ether and the 2,4-dinitrophenylhydrazone isolated by preparative thin layer chromatography and crystallized from a mixture of

petroleum ether (40°-60°)-ether (70:30) v/v. Recovery 58% m.p. 69°-70° (uncorrected) Anal. calculated for $C_{24}H_{40}N_4O_6$, C, 60.0%, N, 11.66%, H, 8.33%, found C, 60.10%, N, 12.05%, H, 8.22%. The 2,4-dinitrophenylhydrazone of the acid was obtained by alkaline hydrolysis of the ester.

The D,2-hydrostearic acid was isolated from the DL (isomers) by conversion to the diastereoisomers with L,(-) ephedrins. After six crystallizations, a crystalline precipitate was obtained, of constant melting point (90°-91°) and constant rotation of $[\alpha]_D^{25}$ $+9.9^{\circ}$ (c 3.4% in methanol). Anal. calcd. for $C_{28}H_{51}NO_4$, C, 72.25%, H, 10.94%, N, 3.04%. Found C, 72.13%, H, 10.82%, N, 2.92%. Recovery, 36%. The salt was decomposed by shaking with a mixture of dilute hydrochloric acid and ether. Recrystallization of the residue from petroleum ether yielded the acid m.p. 95°-96° $[\alpha]_D^{20}$ +3.86 (c 5.46% in pyridine). This acid is of the D configuration (8).

Assay of the Oxidation of the 2-hydroxystearate-1-C^{14} to 2-ketostearic Acid-1-C^{14}. Substrates were solubilized with the aid of Tween 20 (5). The specific activity of the D and DL,2-hydroxystearate-1-C^{14} was 53,000 cpm per 100 mμmoles. The assay mixture contained in one ml 0.5 ml of the 104000xg supernatant of rat kidney homogenate in 0.25 M sucrose (about 1.5 mg protein), NAD^+, $2x10^{-3}$ M, Tween 20, 0.5 mg and tris buffer pH 7.5, $3x10^{-2}$M. The concentrations of the substrates are shown in the experimental section. Incubation was performed in air atmosphere, usually for 20 minutes. The reaction was stopped by addition of 0.1 ml of a saturated solution of 2,4-dinitrophenylhydrazine in 3N HCl, the mixture was allowed to stay at room temperature for 30 minutes and then extracted (9). The extract was chromatographed on a silica gell G plate (Merck-Darmstadt) with chlorophorm-methanol-acetic acid-H_2O (90:10:2:1) v/v. The radioactive spots were located by autoradiography and the silica of the spot corresponding to the 2,4-dinitrophenylhydrazone of the 2-keto acid was scraped from the plate, transferred to a counting vial, containing 10 ml of 0.3% POPOP and 5% PPO in toluene-ethanol (1:1) v/v, and counted in a liquid scintillation counter. Substrate blanks with boiled enzyme were processed in the same manner as the

experimental samples. Counts were corrected for blank values and quenching effect.

Assay of Decarboxylation of the 2-hydroxy Fatty Acids. The assay mixture was the same as described above, except that microsomal fractions in 0.05 ml sucrose 0.25 M and ATP $1x10^{-3}$ M were added. The incubation took place in flasks provided with CO_2 collecting vials (6).

RESULTS AND DISCUSSION:

Characterization of the 2-ketostearic Acid as the Product of the Enzymatic Reaction. The assay mixture, following incubation, was acidified and extracted according to Folch et al. (10). The extract was analyzed by thin layer chromatography and autoradiography before and after methylation with diazomethane. The results shown in Fig. 1 indicate that the reaction product (Fig. 1a) upon methylation, moves at a higher Rf than the methyl ester of the 2-hydrostearate (Fig. 1b). The radioactivity of this spot, examined by two-dimentional thin layer chromatography, was found to be unseparable from the synthetically prepared methyl ester of the 2-keto stearate. In another experiment, the methyl ester of the 2-ketostearate-1-C^{14} was isolated, diluted with non-radioactive material and the 2,4-dinitrophenylhydrazone of the keto ester and the keto acid were prepared as described in Methods. Table 1 shows that the specific radioactivities of the three derivatives of the 2-ketostearate are close to those expected for 2-ketostearate-1-C^{14}, being the product of oxidation. The identity of the 2-ketostearate, isolated from an assay performed with DL,2-hydroxystearate--9,10-H^3 was also established by gas radiochromatography (5). It was therefore concluded that the kidney cell supernatant acquires an oxidase system oxidizing the 2-hydroxy fatty acids to the corresponding 2-keto fatty acids. The assay of the activity of this system was based on the finding that treatment of the reaction mixture with 2,4-dinitrophenylhydrazine in 3N HCl converts all 2-ketostearic acids into 2,4-dinitrophenylhydrazone, which is extracted into $CHCl_3$:CH_3OH (2:1) v/v and, distinctly, separated from the 2-hydroxystearate by thin layer chromatography (Fig. 1c). The radioactivity of this spot is

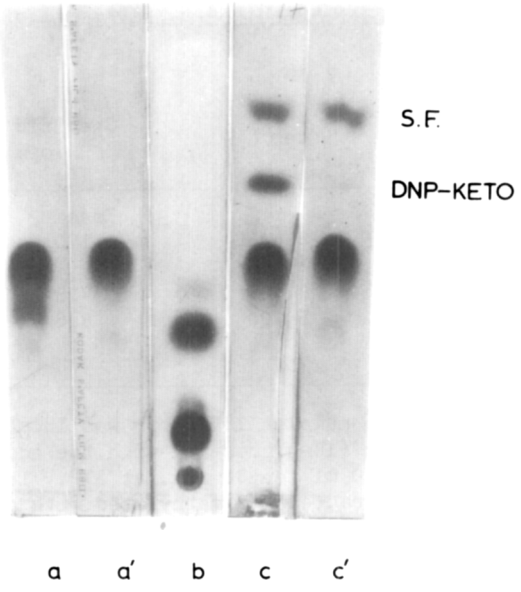

Figure 1. Autoradiogram of thin layer chromatograms showing the product of oxidation of DL-2-hydroxystearate-1-C^{14} in the 104000xg kidney supernatant. (a) Total extract of the assay mixture showing from top: the non-reacted substrate, the product of the enzymatic reaction. (a') Assay performed with boiled enzyme. (b) Same as (a) following methylation with diazomethane, showing from top: the methyl esters of the 2-keto stearate, the non-oxidized substrate. (c) and (c') same as (a) and (a') extracted following 2,4-dinitrophenylhydrazine treatment. Solvent systems for (a) and (c): chloroform-methanol-acetic acid-H_2O (90:10:2:1) v/v; for (b) petroleum ether (40°-60°)-ether-acetic acid (85:15:1) v/v.
(DNP-KETO) is the 2,4-dinitrophenylhydrazone of the 2-ketostearic acid.
The spots of (c) and (c') in the solvent from (S.F.) are artifacts produced during 2,4-dinitrophenylhydrazine treatment. These spots are not produced when further purified enzyme preparations are used.

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unseparable from the spot produced by the 2,4-dinitrophenylhydrazone of the synthetically prepared 2-ketostearic acid. Boiled enzyme does not produce 2-ketostearic acid, (Fig. 1a', c').

Separation of the D and L 2-hydroxy Fatty Acid Oxidases. The 104000xg supernatant of rat kidney homogenates was fractionated with ammonium sulfate; most of the activity was found in the fraction precipitating between 35% and 55% saturation. This enzyme fraction could be separated into two active peaks by DEAE cellulose chromatography (Fig. 2). The enzymatic activities of peak I, oxidized the D isomer and, to a lower extent, equal concentration of the DL

Table 1. Identification of the oxidation product of the DL,2-hydroxystearate-1-^{14}C as the 2,4-dinitrophenylhydrazone of the 2 ketostearate.

	Weight mg	cpm	Specific Radioactivity cpm per mg	
			Found	Expected
2-ketostearate methyl ester	11.4	13,400	1,170	
DNP-keto ester	10.6	8,300	785	740
DNP-keto acid	7.1	5,700	805	775

D,L-2-hydroxystearate-1-C^{14} 375 mμmoles 166000cpm was incubated with 5 ml of 104000xg rat kidney supernatant (16mg protein) with the addition described in the text in a total volume of 10 ml for 30 min. at 37°. The reaction mixture following acidification was extracted and methylated with diazomethane. The 2-ketostearate methyl ester was isolated by thin layer chromatography, diluted with 11.0 mg of the synthetic ketoester and transformed to the 2,4-dinitrophenylhydrazone of the ester (DNP-keto ester) and of the acid (DNP-keto acid) as described in Methods.

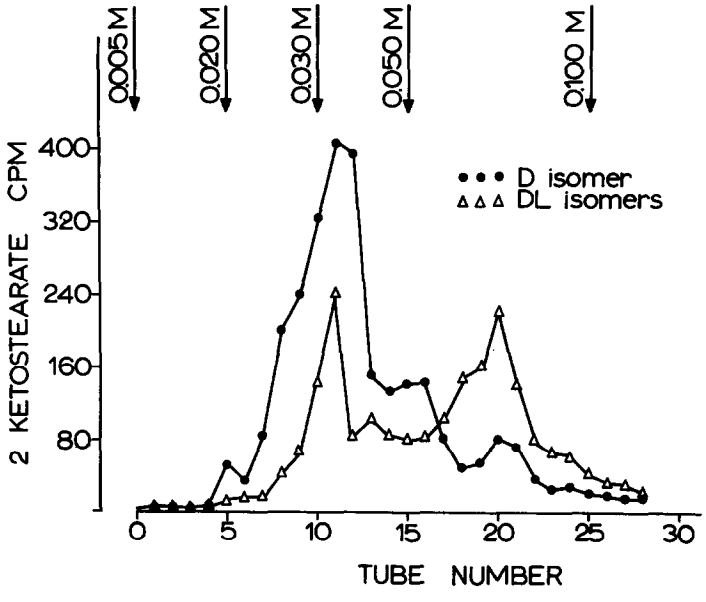

Figure 2. Elution of the D and L,2-hydroxy fatty acid oxidases from DEAE-cellulose. Eight ml of the (35-55%) ammonium sulfate fraction (195 mg protein) were applied on the column which was 2.8x11 cm, equilibrated with 0.005 M phosphate buffer pH 7.4,20 ml fractions were collected. Two half ml of each fraction (0.1-0.4 mg protein) were assayed for oxidase activity each with 75 mμmoles of D or DL isomers.
Peak I is eluted with 0.020 to 0.030 M phosphate buffer and Peak II with 0.03 to 0.05 M.

mixture, suggesting specificity for the oxidation of the D isomer. The variations in the ratio of activities in the different fractions of this peak is suggestive for the presence of more than one oxidase activity. Peak II, oxidized the DL mixture and, to a much lower extent, the D isomer, suggesting specificity of the enzyme for the oxidation of the L isomer. The fractions of the two active peaks were pooled, concentrated by precipitation with 55% saturated ammonium sulfate, dialysed and the kinetics of the reactions catalyzed by the two enzymes were studied. It was found that oxidation of the D isomer by Peak I and of the L isomer (DL form) by Peak II were linear for 20 minutes. The results from substrate concentration experiments have confirmed that Peaks I and II comprise each an oxidase specific for the oxidation of the D and L,2-hydroxystearate respectively and that Peak I is not homogeneous. Omission of NAD^+ from the assay mixture decreased the activity of the two oxidases to approximately 60 percent.

Decarboxylation of the 2-ketostearate. As shown in Fig. 3, the 2-ketostearate formed in the 104000xg supernatant is decarboxylated when the microsomal fraction and ATP are added to the assay mixture. Further experiments have shown that the 2-hydroxy fatty acids can be decarboxylated in the microsomal fraction plus the dialysate of the supernatant or ascorbate. The kinetics of this system, indicated stereospecificity in the decarboxylation of the 2-hydroxy fatty acids. It may therefore be suggested that the oxidases identified in the soluble part of the cell occur also bound to the microsomal fraction.

The physiological significance of the D-2-hydroxy fatty acid oxidase for the degradation of the 2-hydroxy fatty acids becomes evident when it is considered that the 2-hydroxy fatty acids which have been identified in biological material are of the D configuration. In addition, it has recently been shown that 2-hydroxy palmitate is formed by degradation of phytosphingosine in the liver (10). The L,2-hydroxy fatty acid oxidase may participate in the a-oxidation system involved in the degradation of the normal and/or branched long

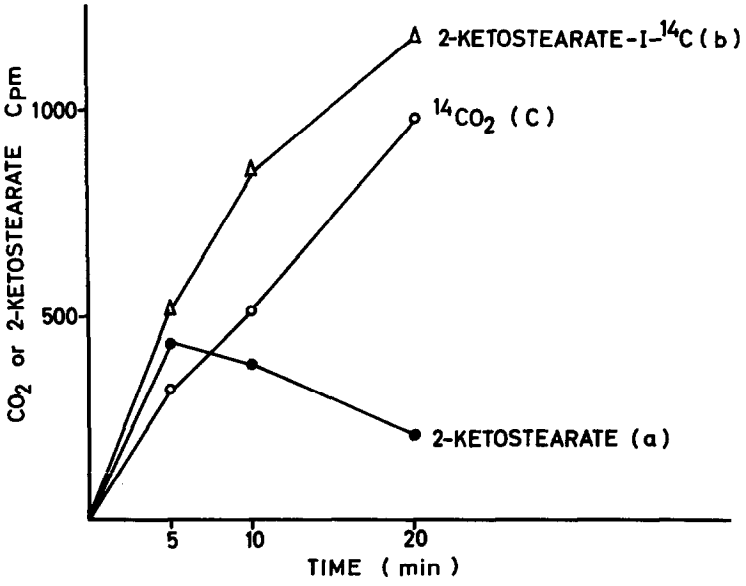

Figure 3. Recovery of 2-ketostearate-1-C^{14} from 2-hydroxystearate-1-C^{14} (750 mμmole per ml) as a function of time of incubation. Assays were performed with the 104000xg supernatant of kidney homogenate, in the presence, or in the absence of the microsomal fraction. $^{14}CO_2$ was produced only when the microsomal fraction was present in the assay mixtures. The results are mean values of duplicates. Correlation of counts resulting from CO_2 and 2-ketostearate was achieved by adjusting the counting conditions to the aid of internal standards.
a-2-ketostearate recovered from the 104000xg supernatant plus microsomes.
b-2-ketostearate formed in the 104000xg supernatant.
C-carbon dioxide.

chain fatty acids (2,11). Strong evidence exists that the L,2-hydroxy fatty acids are intermediates in the a-oxidation of normal fatty acids in plants (3).

The activity of the oxidases presented here seem to depend on reduced pyridine nucleotides. Two aliphatic L-a-hydroxy acid oxidases, which are flavoprotein enzymes, have been isolated from the cortex of hog kidney mitochondria (12); a third, similar enzyme has been isolated from light rat liver mitochondria (13). The soluble L,2-hydroxy fatty acid oxidase described here, differ from these enzymes in both their specificity with regard to the chain length of the substrates and their cofactor requirements. Apparently, no enzyme with the specificity of the D,2-hydroxy fatty acid oxidase herein described, has been reported previously.

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