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THE BIOLOGICAL OXIDATION OF 2,2-DIMETHYLOCTANOIC ACID

HALINA DEN*
Department of Biological Chemistry, Medical School, The University of Michigan, Ann Arbor, Mich. (U.S.A.)
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SUMMARY

The administration of 2,2-dimethyl[1-^{14}C]octanoic acid to rats results in the urinary excretion of four ^{14}C-labeled polar metabolites. Three of these have been identified as 2,2-dimethyladipic acid, 2,2-dimethyl-7-hydroxyoctanoic acid, and 2,2-dimethyl-7-ketooctanoic acid.

The same products are also formed during the incubation of 2,2-dimethyloctanoate with rat-liver slices.

These results suggest that either the initial oxidative attack is not specific for the ω-position, or that ω- and ω-1-oxidation products are interconverted enzymically.

INTRODUCTION

It has been known since the classical experiments of VERKADE[1] that the animal organism can accomplish the ω-oxidation of fatty acids, and there are now many examples of ω-oxidation of fatty acids containing α- or β-substituents or with a modified carboxyl group[2-9]. The process of ω-oxidation has also been studied in cell-free systems. ROBBINS[10] reported the conversion of C_8 to C_{12} monocarboxylic acids to the corresponding dicarboxylic acids in tissue homogenates, and WAKABAYASHI AND SHIMAZONO[11,12] demonstrated the conversion of sorbic acid amide to the ε-hydroxy compound and muconic acid amide as well as the conversion of the amides of octatrienoic acid and of capric acid to the corresponding dicarboxylic acid monoamides. Both groups presented evidence for the microsomal hydroxylation of the terminal methyl group with a requirement for TPNH and molecular oxygen. KUSUNOSE, KUSUNOSE AND COON[13] have recently demonstrated the ω-oxidation of even-numbered fatty acids from C_8 to C_{18} in a bacterial enzyme system. The ω-hydroxylation reaction requires molecular oxygen, DPNH, Fe^{2+}, and two soluble enzymes. The ω-hydroxy

* Present address, Department of Biochemistry, School of Medicine, Indiana University, Indianapolis, Indiana. Portions of the data in this paper are taken from a thesis submitted by the author in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biological Chemistry in the Rackam School of Graduate Studies of The University of Michigan.

fatty acids are further converted to the dicarboxylic acids by DPN-dependent dehydrogenases.

Our choice of 2,2-dimethyl[1-^{14}C]octanoic acid as a substrate for the study of ω-oxidation was prompted by the work of BERGSTRÖM *et al.*[7] who showed that the administration of 2,2-dimethyl[1-^{14}C]stearate to rats led to the excretion of 60–90% of the administered isotope in the urine in the form of 2,2-dimethyl[1-^{14}C]adipate.

In the present paper evidence is presented for the formation of radioactive 2,2-dimethyl-7-ketooctanoate and 2,2-dimethyl-7-hydroxyoctanoate as well as 2,2-dimethyladipate as metabolites of 2,2-dimethyl[1-^{14}C]octanoate. The formation of such products thus provides evidence for both ω- and "ω-1" oxidation.

METHODS

2,2-Dimethyloctanoic acid and related compounds

2,2-dimethyl[1-^{14}C]octanoic acid was synthesized according to the following reactions:

$$(CH_3)_2CH^{14}CO_2H \rightarrow (CH_3)_2CH^{14}COCl \rightarrow (CH_3)_2CH^{14}CO_2C(C_2H_5)_3 \rightarrow$$
$$\rightarrow CH_3(CH_2)_5C(CH_3)_2{}^{14}CO_2C(C_2H_5)_3 \rightarrow CH_3(CH_2)_5C(CH_3)_2{}^{14}CO_2H.$$

Carboxyl-labeled isobutyric acid was prepared from 6.2 g of isopropyl bromide and an equimolar amount of $^{14}CO_2$ by the Grignard reaction according to the general method of SAKAMI *et al.*[14]. The product was distilled at atmospheric pressure to give 2.1 g of the acid, representing 47% of the theoretical yield. The labeled acid was diluted with 6.7 g of carrier and treated with $SOCl_2$, and the reaction mixture was submitted to distillation to furnish 9.7 g of [1-^{14}C]isobutyryl chloride (91% yield). The acid chloride was converted to the desired product according to a general method for the preparation of trialkylacetic acids described by HAUSER AND CHAMBERS[15]. To a suspension of 3.9 g of potassium in anhydrous ether were added 10.5 g of triethylcarbinol prepared by a modification[15] of the method of LUCAS[16]. When the metal had reacted, the labeled isobutyryl chloride dissolved in anhydrous ether was added dropwise, and the mixture was boiled under reflux for 3 h. From the reaction mixture 8.0 g of triethylcarbinyl isobutyrate, a colorless liquid, were obtained (b.p. 70–72° at 9 mm); the yield was 47% of the theoretical amount. The labeled ester was dissolved in ether and added dropwise with stirring to a solution of 2 g of potassium in 250 ml of liquid NH_3. The resulting mixture was stirred for 50 min, 7.1 g of redistilled hexyl bromide dissolved in ether were added dropwise, and stirring was continued for an additional hour. Distillation of an ether solution of the crude product furnished 7.0 g of triethylcarbinyl dimethyloctanoate (b.p. 116–118° at 3 mm), representing a 61% yield. The ester was hydrolyzed with conc. HCl in dioxane, and an ether solution of the product was distilled to give [1-^{14}C]-2,2-dimethyloctanoic acid, a colorless liquid, in 78% yield (b.p. 126° at 7 mm). The acid, which had a specific activity of $1.3 \cdot 10^4$ counts/min per mg, gave only one spot, R_F 0.79, upon paper chromatography in *n*-butanol–water–diethylamine (100:15:1, v/v.) (ref. 17) (Found: C, 69.71; H, 11.74. $C_{10}H_{20}O_2$ requires C, 69.70; H, 11.72%).

2,2-Dimethyl-7-ketooctanoic acid was synthesized by a similar method to that employed for dimethyloctanoic acid. 6-Bromo-2-hexanol was prepared according to the method of ANDERSON AND CRAWFORD[18], and was converted to the corresponding

ethylene ketal (b.p. 112° at 14 mm) by the general procedure described in ref. 19. The alkylation of 9.3 g of triethylcarbinyl isobutyrate by 11.0 g of the ethylene ketal of 6-bromo-2-hexanone gave 7.3 g of product (b.p. 137–139° at 0.5 mm), which upon hydrolysis gave 2.6 g of the keto acid (b.p. 135° at 0.8 mm). (Found: C, 63.96; H, 9.72. $C_{10}H_{18}O_3$ requires C, 64.48; H, 9.74%). Reduction of the keto acid with $NaBH_4$ according to the method of REID AND SIEGEL[20] gave 2,2-dimethyl-7-hydroxyoctanoic acid in quantitative yield.

Non-radioactive 2,2-dimethyloctanoic acid was made according to the same procedure or by the method of HOI AND COGNIANT[21]. 2,2-Dimethylsuccinic acid was made by the method of VOGEL[22] as modified by BERGSTRÖM *et al.*[7] and 2,2-dimethyladipic acid was kindly furnished by Dr. W. STEELE.

Column chromatography

For the separation of dimethyloctanoic acid from its metabolic products column chromatography was employed as described by HOWARD AND MARTIN[23] with a slight modification of the solvent systems described by BERGSTRÖM *et al.*[7] A mixture of 15 ml of chloroform, 15 ml of 2-ethylhexanol, 120 ml of absolute methanol and 180 ml of water was allowed to cool to room temperature, and the two layers were separated. For preparation of the column, 3-g portions of hydrophobic kieselguhr (Hyflo Supercel treated with dichlorodimethylsilane) were mixed with 2.6 ml of the lower (chloroform-rich) phase, about 20 ml of the upper phase were added, and the resulting slurry was transferred to a column 11 mm in diameter. To the packed column, of final length about 10 cm, was applied 1 ml of the upper phase to which the radioactive acids have been added. The column was developed by passage of additional amounts of the upper phase, and 2.5-ml fractions were collected. Under these conditions 2,2-dimethyloctanoic acid remains on the column and may be eluted with 75% aq. methanol.

RESULTS

Conversion of 2,2-dimethyloctanoate to polar acids in intact animals

A solution of 60 mg of radioactive 2,2-dimethyloctanoic acid in 1 ml of olive oil was administered by gastric intubation to each of several adult male rats. About 45% of the administered ^{14}C was recovered in the urine excreted in the following 24 h and about 10% in the subsequent 48-h period. When the urine was acidified to pH 1 with H_2SO_4 and extracted continuously with ether for 8 h, the bulk of the radioactivity was removed. In order to extract most of the remaining radioactivity from the acidified aq. layer it was found necessary to boil it under reflux for 1 h prior to extraction with ether. This finding is in accord with the observations of BERGSTRÖM *et al.*[7] who found that the urinary metabolites arising from 2,2-dimethylstearate are excreted partly in conjugated forms, possibly as glucuronides. The combined ether extracts were dried over anhydrous sodium sulfate and concentrated to a small volume, and aliquots were submitted to paper chromatography in *n*-butanol–water–diethylamine (100:15:1, v/v.) followed by autoradiography. As illustrated in Fig. 1, three radioactive products have been detected (R_F 0.14, 0.29 and 0.64) in addition to the residual 2,2-dimethyloctanoic acid (R_F 0.79). Cochromatography with an authentic sample on paper established the identity of the most polar compound

(R_F 0.14) as 2,2-dimethyladipic acid. Radioactive 2,2-dimethylsuccinic acid could not be detected in the urinary extract.

In attempts to determine the nature of the compound of R_F 0.64 this area was eluted from several paper chromatograms with 0.01 N methanolic NaOH, and the combined eluates were acidified and extracted with ether. The ether was removed, and the residue was dissolved in appropriate solvents and found to give no reaction with bromine water or permanganate. On the other hand, treatment with dinitrophenylhydrazine in HCl resulted in the formation of a product which upon extraction into alkali gave a purple color, thereby indicating the presence of a carbonyl group in the compound. A portion of the residue was treated with sodium hypoiodite. The iodoform generated was recrystallized from methanol and identified by melting point. These results were taken to indicate that the unknown acid was 2,2-dimethyl-7-ketooctanoate. In support of this conclusion the R_F value of the unknown acid (0.64) was found to be identical to that of the chemically synthesized 2,2-dimethyl-7-ketooctanoic acid (0.64) (Fig. 1).

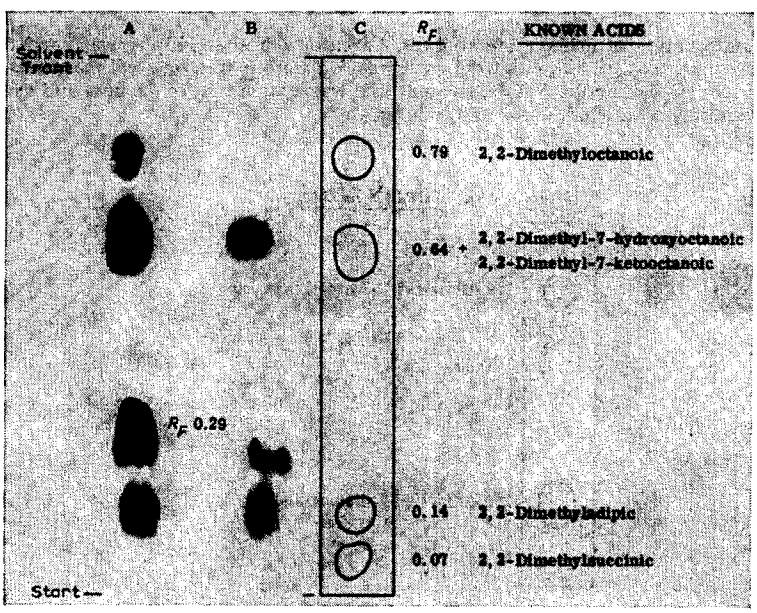

Fig. 1. Paper chromatography of the oxidation products of 2,2-dimethyloctanoic acid. Solvent: n-butanol–water–diethylamine (100:15:1, v/v.) (ascending). The acids were detected by spraying with 0.5% bromthymol blue in ethanol and by autoradiography. A, urine; B, liver slice incubation medium from which unchanged 2,2-dimethyloctanoic acid was removed by passage of the extracted acids through the siliconized Hyflo-Supercel column; C, mixture of chemically synthesized standards.

As indicated in Fig. 2, radioactive acids extracted from rat urine yielded two distinct radioactive peaks when submitted to column chromatography on siliconized Hyflo-Super-cel. Analysis of these peaks was effected by submitting the column effluent fractions No. 2 through 13 (Peak I) and No. 16 through 30 (Peak II) to paper chromatography in n-butanol–water–diethylamine. 2,2-Dimethyladipic acid (R_F 0.14)

and the metabolite of R_F 0.29 were present in Peak I. Peak II yielded one spot having R_F value of 0.64.

About 92% of the isotope applied to the column was recovered in the effluent fractions No. 2 through 30 and was distributed about equally between the two peaks I and II. The remaining 8% of the isotope could be eluted from the column with aq.

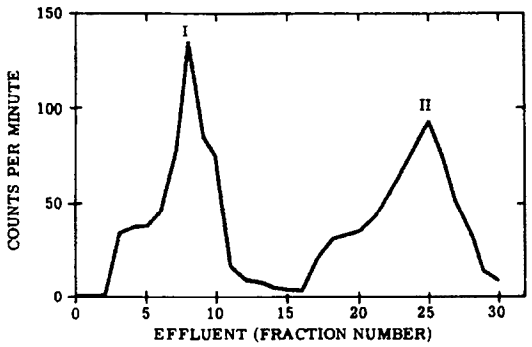

Fig. 2. Column chromatography of urinary metabolites. Partition chromatography was carried out as described in the text. 0.05-ml aliquots from each fraction were plated on steel planchets, containing 0.02 ml of 0.01 N methanolic NaOH, the material was evaporated to dryness and the radioactivity was determined in a window-gasflow counter.

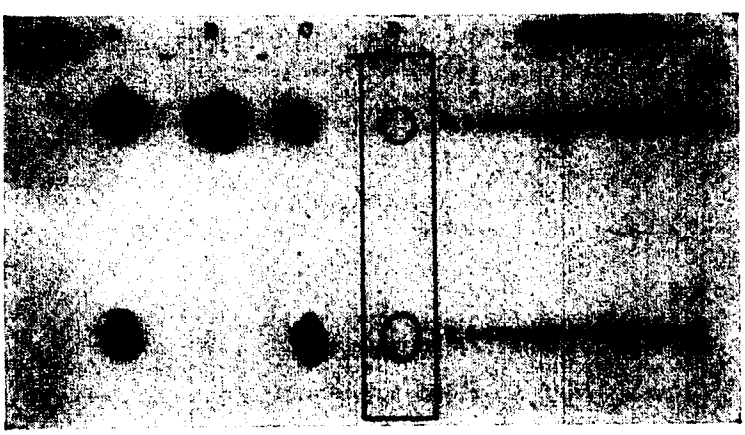

Fig. 3. Resolution of Peak II by descending chromatography in methanol–satd. heptane. The acids were detected by spraying with 0.04% bromcresol green in ethanol, adjusted to pH 7 with NaOH, and by autoradiography. A, urine; B, urine after reduction with $NaBH_4$; C, liver slice incubation medium; D, known compounds.

75% methanol. Identity of this material as the residual 2,2-dimethyl[1-^{14}C]octanoic acid was established by paper chromatography (R_F 0.79).

As shown in Fig. 1, authentic 2,2-dimethyl-7-ketooctanoic acid and 2,2-dimethyl-7-hydroxyoctanoic acid are indistinguishable when subjected to paper chromatography in *n*-butanol–water–diethylamine. Fig. 3 depicts a chromatogram of Peak II illustrating resolution of this material into two components by methanol–satd. heptane[14]. The two radioactive compounds separated by this solvent exhibited

chromatographic properties identical to those of the chemically synthesized 2,2-dimethyl-7-hydroxyoctanoic acid and 2.2-dimethyl-7-ketooctanoic acid. Estimation of spot-intensities on radioautographs indicates that the amount of 2,2-dimethyl-7-hydroxyoctanoic acid exceeds by far the amount of the corresponding keto acid.

To obtain a derivative of 2,2-dimethyl-7-ketoooctanoic acid, fractions 16 through 30 from 12 separate columns (about 10^6 total counts/min) were pooled, and taken to dryness *in vacuo*. The residue was dissolved in a minimal amount of 95% ethanol and treated with 2,4-dinitrophenylhydrazine in 1 N HCl. The isolated phenylhydrazone was recrystallized to constant radioactivity by the addition of water to a solution in the minimal amount of 95% ethanol. The specific activities upon successive crystallizations were as follows: 6300, 6700, 6680 and 6690 counts/min per mg of compound. The final product, which was dried *in vacuo* over P_2O_5, had a sharp melting point (131–132°, corr.). (Found, C, 52.47; H, 5.99; N, 15.24. $C_{16}H_{22}N_4O_6$ requires C, 52.44; H, 6.06; N, 15.29%).

The dinitrophenylhydrazone prepared from the chemically synthesized keto acid had a melting point of 131–132° (corr.) alone or mixed with the derivative of the biologically formed keto acid, and the infrared spectra were essentially identical.

Products of 2,2-dimethyloctanoate oxidation by liver slices

Rat-liver slices (2.5 g, wet wt.) were incubated with 20 ml of Krebs–Ringer bicarbonate buffer and 3 ml of 0.1% potassium 2,2-dimethyl[1-^{14}C]octanoate at 37° for 3 h under air. The incubation mixture (after removal of liver slices) was subjected to copper-lime treatment according to the method of LEHNINGER AND SMITH[25]. The resulting precipitate was washed with water and the combined supernatant fluids were acidified to pH 1 and extracted with ether.

Paper chromatography of this extract in *n*-butanol–water–diethylamine gave the same products (R_F 0.14, 0.29 and 0.64) as previously detected in urine (Fig. 1). Also, as illustrated in Fig. 3, chromatography of column Peak II in methanol–satd. heptane showed the presence of greater amount of 2,2-dimethyl-7-hydroxyoctanoic acid than 2,2-dimethyl-7-ketooctanoic acid.

In another experiment the incubation mixture contained 500 mg of liver slices, 5 ml of Krebs–Ringer bicarbonate buffer and 4.0 μmoles ($4 \cdot 10^5$ counts/min) of potassium 2,2-dimethyl[1-^{14}C]octanoate (specific activity of this preparation was $5.8 \cdot 10^5$ counts/min per mg of acid.) Incubation was performed by shaking for 3 h in air at 37° and was terminated by addition of 5 ml of 50% KOH and 3 ml of methanol. The saponification was completed by heating for 30 min in a water bath at 85°. After removal of the nonsaponifiable lipid by extraction with pentane the reaction mixture was acidified and extracted with ether. The ether extract was washed, dried over $MgSO_4$ and the solvent was removed. Onefourth of the residue (about 96000 counts/min) was subjected to column chromatography on siliconized Hyflo-Supercel. About 43% (42000 counts/min) of the isotope was recovered in the effluent fractions No. 2 through 30. (In a control experiment with boiled slices only about 100 counts/min were found in these fractions). About 57% (53000 counts/min) was eluted from the column with 75% aq. methanol in the form of unchanged substrate. Thus, of the 4.0 μmoles of 2,2-dimethyl[1-^{14}C]octanoate added to the incubation mixture about 1.7 μmoles were converted to polar acids.

DISCUSSION

The results presented indicate that 2,2-dimethyl-7-hydroxyoctanoic acid, 2,2-dimethyl-7-ketooctanoic acid, and 2,2-dimethyladipic acid are products of the biological oxidation of 2,2-dimethyloctanoic acid. Additional evidence for the formation of these products as well as 2,2-dimethyl-8-hydroxyoctanoic acid and 2,2-dimethylsuberic acid has been recently obtained by gas-liquid chromatography[26]. To the author's knowledge the present work and the recent report of PREISS AND BLOCH[27] provide the only examples of ω-1 oxidation of carboxylic acids in animal system. Occurrence of ω-1 fatty acid oxidation in yeast was previously reported by TULLOCH *et al.*[28].

Other types of compounds possessing alkyl chains have also been shown to undergo oxidation on both the terminal and penultimate carbon atoms, such as alkylbenzenes[29], barbiturates[30], and hydrocarbons[31]. Since the nature of the enzymatic oxidation of these various substrates is not known, the significance of the occurrence of both ω-and ω-1 oxidation is a matter of speculation. It appears, however, that the initial oxidative attack is not specific for the ω-position since it has been found recently[26] that rat liver microsomes fortified with a TPNH-generating system catalyze hydroxylation of 2,2-dimethyloctanoic acid at both ω- and ω-1 positions.

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