THE EFFECT OF 4-PENTENOIC ACID ON FATTY ACID OXIDATION.[1]

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4-Pentenoic acid has been found to inhibit gluconeogenesis from three carbon precursors both in vitro and in vivo (1-4). It has also been shown to inhibit fatty acid oxidation in heart and liver (1, 2). In recent communications from this laboratory, it was suggested that the inhibitions of gluconeogenesis and fatty acid oxidation by 4-pentenoic acid were due to depressed tissue levels of coenzyme A and (-)-carnitine (5, 6), which are important cofactors in both of these inhibited processes (7, 8, 9). In this communication, we present data which show: (a) the 4-pentenoate-induced inhibition of fatty acid oxidation in pigeon liver homogenates is augmented by preincubation with the inhibitor, (b) the inhibition of fatty acid oxidation in pigeon liver homogenates is associated with a decrease in the levels of coenzyme A and (-)-carnitine, (c) the incubation of pigeon liver homogenates with 4-pentenoic acid results in its oxidation, and the accumulation of 4-pentenoyl, acryloyl and acetyl carnitines, (d) the 4-pentenoic acid-induced inhibition of fatty acid oxidation in pigeon liver homogenates can be prevented by the addition of appropriate amounts of CoA and (-)-carnitine.

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MATERIALS AND METHODS

Two to three-month old white pigeons obtained from the Palmetto Pigeon Farms of South Carolina were fasted for 50 hours prior to sacrifice. The animals received water ad lib during the fasting period. The animals were decapitated and the liver immediately excised, chilled in ice cold calcium-free Krebs-Ringer Phosphate buffer pH 7.4, and homogenized in 9 volumes of the same solution which had previously been gassed with oxygen. Determination of $C^{14}O_2$ production from palmitate-1-C^{14}, pentanoate-1-C^{14} and 4-pentenoate-1-C^{14} were carried out essentially as previously described (10).

Concentrations of free carnitine in pigeon liver homogenates were determined by termination of the incubation with trichloroacetic acid and centrifugation to obtain a supernatant, which was exhaustively extracted with diethyl ether. The aqueous extract was lyophilized and a methanolic extract made of the dried residue. The alcoholic extract was evaporated to dryness and this residue taken up in water and used in the acyl coenzyme A-carnitine-acetyltransferase assay by the procedure of Tubbs, Chase and Pearson (11). Coenzyme A-carnitine-acetyltransferase was obtained commercially (Boehringer).

Coenzyme A and acyl CoA levels were determined by a procedure to be described in more detail in a forthcoming publication[4]. Tracer amounts of coenzyme A-pantetheine-H^3 were added to pigeon liver homogenate incubates containing 1 μmole/ml reduced glutathione. The incubation was terminated by the addition of methanol to the reaction mixture. The reaction mixture was centrifuged and the supernatant passed through 0.2 cm^2 x 3 cm columns of a mercuriated phenol-formaldehyde resin coated on silica gel similar to a procedure of Miles (12). The effluents from these columns charged with sulfhydryl group specific material, and the column washings were combined and evaporated to dryness. The residue was taken up in methanol-water added to toluene-triton x 100 scin-

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tillation cocktail and counted in a liquid scintillation spectrometer. The ratio of counts held on the column to counts in the effluent, corrected for counts present in the zero time incubation effluent, gives a measure of the ratio of the labeled free and labeled acyl CoA in the incubation mixtures.

4-Pentenoic acid-1-C^{14}, 4-pentenoic acid-4,5-H^3, 4-pentenoyl carnitine and acryloyl carnitine were synthesized (1, 2, 13). Pentanoate-1-C^{14} was prepared from 4-pentenoate-1-C^{14} by catalytic hydrogenation. (-)-Carnitine was prepared as previously described (13). 4-Pentenoyl coenzyme A was synthetized after the procedure of Wieland and Koeppe (14). Acryloyl coenzyme A was prepared from acrylic anhydride by the procedure of Simon and Shemin (15). Carnitine and acylcarnitines were separated on Al_2O_3 plates (Brockman IV), using chloroform-methanol-ammonia-water (12:7:1:1). Coenzyme A-pantetheine-H^3 was a gift of Dr. P. Majeras of the Department of Medicine, Washington University of St. Louis.

RESULTS

Figure 1 shows the conversion to $C^{14}O_2$ of palmitate-1-C^{14} added to pigeon liver homogenates after varying periods of preincubation of the homogenates without additions (endogenous), with pentanoic acid or with 4-pentenoic acid. The incubations with endogenous substrate show a similar time course of palmitate oxidation as the incubations to which pentanoic acid was added, whereas the rates of oxidation were higher in the absence than in the presence of pentanoic acid. Homogenates which had been preincubated with 4-pentenoic acid showed a marked decrease in palmitate-1-C^{14} oxidation. The magnitude of the depression of palmitate oxidation varied with the period of the preincubation and reached approximately 80% after 15 minutes. The addition of CoA and (-)-carnitine to incubates containing no 5-carbon acid or containing pentanoate resulted in similar magnitudes of stimulation of palmitate-1-C^{14} oxidation. Addition of CoA and carnitine to the reaction mixtures incubated with 4-pentenoic acid resulted in partial prevention of the inhibitory effects of 4-pentenoate on palmitate-1-C^{14} oxidation.

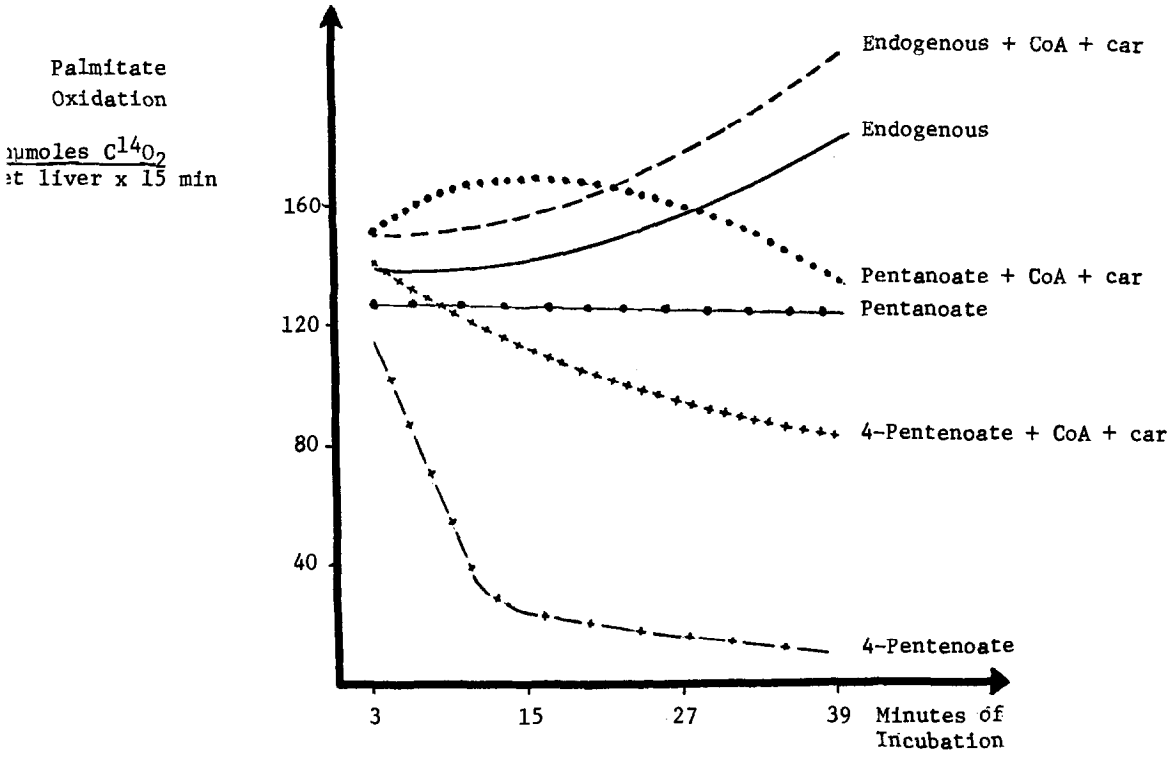

Figure 1. Palmitic Acid Oxidation by Pigeon Liver Homogenate in Presence of 5-Carbon Acids. The incubations contained 2 ml 10% pigeon liver homogenate in calcium free KRP buffer of pH 7.4. No additions were made, or 1 µmole of 4-pentenoate or 1µmole of pentanoate and where indicated 200 mµmoles coenzyme A and 400 mµmoles (-)-carnitine were added at the beginning of the 55-minute, 40° incubation. 200 mµmoles palmitic acid-1-C^{14} (200,000 cpm) was added at 3, 15, 27, 39 minutes after the beginning of the experiment and $C^{14}O_2$ was collected for 15 minutes.

Figure 2 shows that 4-pentenoic acid not only inhibited long-chain fatty acid oxidation, but also depressed its own oxidation. The addition of 4-pentenoic acid-1-C^{14} to pigeon liver homogenates after varying periods of preincubation with non-labeled 4-pentenoic acid resulted in a time dependent inhibition of 4-pentenoate-1-C^{14} oxidation to $C^{14}O_2$. About 40% depression of oxidation was found after 15 minutes of preincubation and approximately 80% depression after 30 minutes. An analogue experiment in which the effect of preincubation with non-labeled pentanoic on pentanoic acid-1-C^{14} conversion to $C^{14}O_2$ was carried out and revealed no depression of oxidation. The addi-

tion of optimal amounts of CoA and carnitine at the start of the incubation stimulated the oxidation of 4-pentenoic acid-1-C^{14} and completely prevented the inhibitory effects of 4-pentenoic acid on its own catabolism. If these results are compared with those of Figure 1, it is apparent that palmitate oxidation is more sensitive to inhibition by 4-pentenoate than is 4-pentenoate oxidation.

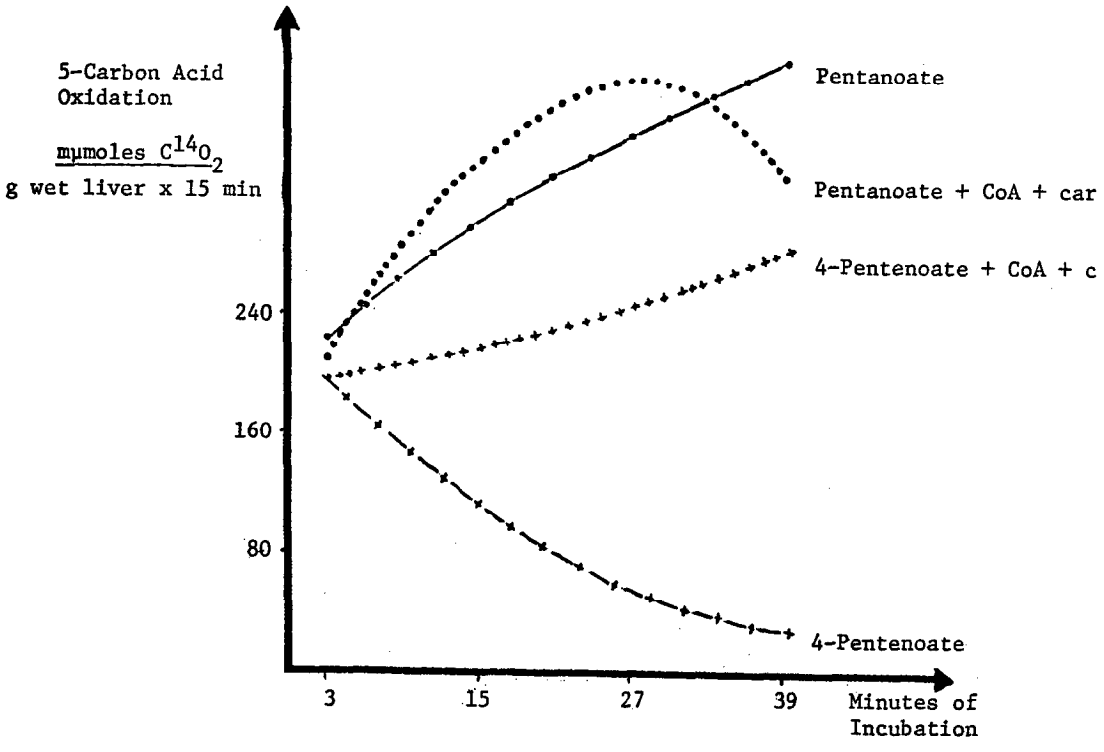

Figure 2. Pentanoic and 4-Pentenoic Acid Oxidation by Pigeon Liver Homogenate. Conditions were as in Figure 1, but instead of palmitate, 500 mμmoles 4-pentenoate-1-C^{14} (150,000 cpm) and pentanoate-1-C^{14} (150,000 cpm) were added as radioactive substrates.

The data of Table 1 show that incubation of pigeon liver homogenates with 4-pentenoic acid resulted in marked changes in the levels of (-)-carnitine, CoA and acyl CoA's. 4-Pentenoic acid caused significant depressions of (-)-carnitine and CoA, whereas it resulted in increased levels of acyl CoA's, and acyl carnitines (data not presented here). Changes of this magnitude in CoA,

(-)-carnitine and their acyl derivatives were not produced by incubations with pentanoic acid, the saturated analogue of 4-pentenoic acid or with endogenous substrate.

TABLE 1.

Carnitine and CoA Levels in Presence of 5-Carbon Acids.

10 ml of a 10% pigeon liver homogenate in calcium free KRP buffer pH 7.4 were incubated at 40° for 60 minutes in the presence of coenzyme A-pantetheine H^3 (100,000 cpm) and in presence and absence of 5 µmoles 4-pentenoic acid or pentanoic acid.

Addition	mµmoles g wet liver (-)-carnitine	% of Total Coenzyme A	
		CoA-SH	Acyl CoA
Control	600 ± 38	83 ± 2%	17 ± 2%
Pentanoic Acid	550 ± 46	79 ± 3%	21 ± 3%
4-Pentenoic Acid	350 ± 33	54 ± 2%	46 ± 2%

TABLE 2.

Formation of Acyl Carnitines in Presence of 4-Pentenoate.

The incubations contained 10 ml 10% pigeon liver homogenate in calcium free KRP pH 7.4, 2.5 µmoles coenzyme A, 5 µmoles (-)-carnitine and 2 µmoles sodium fumarate. Where appropriate 10 µmoles 4-pentenoic acid-1-C^{14} (1 x 10^6 cpm), 10 µmoles 4-pentenoic acid -4,5-H^3 (1 x 10^6 cpm) or 2 µmoles palmitic acid were added. Incubations were carried out at 40° for 30 minutes.

Additions	Acetyl Carnitine	Acryloyl Carnitine	4-Pentenoyl Carnitine
	cpm x 10^4 /g wet weight liver.		
4-Pentenoic Acid-H^3	--	9	18
4-Pentenoic Acid-H^3 + Palmitate	--	13	13
4-Pentenoic Acid-C^{14}	11	--	16
4-Pentenoic Acid-C^{14} + Palmitate	10	--	8

Incubations of pigeon liver homogenates containing optimal concentrations of CoA and (-)-carnitine with 4-pentenoic acid-1-C^{14} or 4-pentenoic acid-4,5-H^3 resulted in the production of various acyl carnitines. The appearance of acryloyl-2,3-H^3 carnitine in incubations containing 4-pentenoic acid 4,5-H^3 indicates that 4-pentenoic acid undergoes β-oxidation. This is further confirmed by the appearance of acetyl-1-C^{14} carnitine in incubations containing 4-pentenoic acid-1-C^{14}. When non-radioactive palmitate was added to incubations containing 4-pentenoate-4,5-H^3, the ratio of acryloyl-2,3-H^3 carnitine to 4-pentenoyl-4,5-H^3 carnitine was increased. Under the same conditions, but using 4-pentenoic acid-1-C^{14} as the precursor, the ratio of 4-pentenoyl-1-C^{14} carnitine to acetyl-1-C^{14} carnitine was decreased.

DISCUSSION

The data presented in this communication show that 4-pentenoic acid inhibits both long and medium-chain fatty acid oxidation in pigeon liver homogenates after a latent period. The effect on long-chain fatty acid oxidation, however, seems to be more pronounced. 4-Pentenoic acid is β-oxidized to acetyl CoA and acryloyl CoA which are substrates of the short-chain acyl CoA-carnitine acyltransferase, and are, therefore, in equilibrium with their respective carnitine derivatives. The formation of 4-pentenoyl, acryloyl and acetyl carnitines from 4-pentenoic acid has been shown by way of tracer studies and these compounds have been identified chemically. The formation of these acyl carnitines is consonant with the decreased levels of free carnitine found in the 4-pentenoate incubates. The fact tha 4-pentenoyl as well as acryloyl carnitine are substrates of the acyl CoA-carnitine acyltransferase also explains the increased levels of acyl coenzyme A and decrease levels of free coenzyme A found in the presence of 4-pentenoic acid. It is noteworthy that the addition of palmitate to 4-pentenoic acid metabolizing pigeon liver homogenates increases the relative amounts of acryloyl carnitine and at the same time decreases the amounts of 4-pentenoyl carnitine suggesting that palmitate augments the translocation of the acryloyl moiety across the mitochondrial membran

and competes with 4-pentenoic acid activation in the extramitochondrial space. The results of our experiments are in agreement with an hypotheses advanced in earlier communications that the deleterious effects of 4-pentenoic acid on fatty acid oxidation are partly due to decreases in the levels of the cofactors carnitine and CoA.

The prevention of the inhibitory effects of 4-pentenoic acid on fatty acid oxidation by addition of CoA and (-)-carnitine are consonant with this hypothesis. Whereas CoA and carnitine addition can prevent the inhibition of fatty acid oxidation by 4-pentenoic acid, they cannot completely reverse an already established inhibition of fatty acid oxidation by 4-pentenoic acid. This may be due to other later effects on fatty acid oxidation such as enzyme inhibition by acryloyl CoA or an effect of 4-pentenoic acid or one of its metabolites on the electron transport chain.

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